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Biotechnological strategies to induce resistance to Sharka in *P. persica* L.

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ABSTRACT

Since the beginning of agriculture, plant virus diseases have been a strong challenge for farming. Following its discovery at the very beginning of the '90s, the RNA interference (RNAi) mechanism has been widely studied and exploited as an integrative tool to obtain resistance to viruses in several plant species, with high target-sequence specificity. In this thesis, the major aspects of Host-induced gene silencing (HIGS), as one of the possible plant defence methods, using genetic engineering techniques have been reviewed. In particular, the use of RNAi-based gene constructs to introduce stable resistance in host plants against viral diseases, by triggering post-transcriptional gene silencing (PTGS) has been described. Recently, Spray-induced gene silencing (SIGS), consisting of the topical application of small RNA molecules (sRNAs) to plants, has been explored as an alternative tool to the stable integration of RNAi-based gene constructs in plants.

Specifically, Sharka disease represents a major threat to peach production worldwide and its etiological agent, plum pox virus (PPV), is included in the Top 10 catastrophic plant virus list. With the final aim to develop strategies to induce PPV resistance in peach exploiting RNAi mechanism, both HIGS and SIGS approaches have been attempted. In particular, with the aim to stable express RNAi-inducing dsRNA against PPV in peach (HIGS strategy), the work was focused on development of *in vitro* protocols for the regeneration and genetic transformation suitable for this species. First of all, an overview of research on the regeneration and genetic transformation in *Prunus persica* L. have been reviewed and novel strategies and procedures aimed at producing transgenic peach lines have been summarized. The results achieved prove that adventitious shoot regeneration seems to be an effective strategy for this species. Even if with different regeneration efficiencies, adventitious shoot regeneration via organogenesis starting from different mature and juvenile tissues of different peach rootstocks and cultivars has been obtained. Differently, the integration of the transgene/s into the plant genome using different species of *Agrobacterium* and then the recovery of transformed plants is extremely difficult. In recent times, the exogenus application of RNAi-inducing dsRNA to plants (SIGS strategy) is considered a promising tool for virus defence for those plant species with high recalcitrance to genetic transformation systems, as peach. With the aim to develop RNAi-based products to control PPV infection in peach, protocols for the production and the topical application of RNAi-inducing dsRNA were also tested. Although the agroinfiltration and the molecules delivered system used have been simple, fast, and cheap, the SIGS approach presented here is at very preliminary step and alternative strategies in terms of both dsRNA production and delivery must be explored in the next future.

In conclusion, HIGS strategy proves to be complicated as virus defence approach in peach plant due to the difficulty in obtaining an efficient transformation system. Thus, the development on new dsRNA-based products to control virus infections, as Sharka for instance, looks an interesting alternative, but several steps (i.e production and delivery methods) need to be developed in order to demonstrate their efficacy.

CHAPTER 1

HOST-INDUCED GENE SILENCING AND SPRAY-INDUCED GENE SILENCING FOR CROP PROTECTION AGAINST VIRUSES[1]

1. Introduction

Plant viruses represent a major threat to global agriculture. Viruses have been found in all cultivated plant species and a wide range of wild species according to the Tenth Report of International Committee on Taxonomy of Viruses (Lefkowitz et al., 2017). Viruses are infectious particles containing a nucleic acid core of RNA or DNA, surrounded by a protective shell made of one or more coat proteins. They are considered as obligate parasites, as they exploit the host cell machinery for their replication in living cells. In particular, during the infection process, a plant virus penetrates the plant cell through wounds made, for example, by arthropod pests or during agricultural practices (for example badly executed pruning); progressively, it colonizes the surrounding cells/tissues and spreads through the whole plant via the phloem. Agricultural practices such as crop rotation, precocious detection and prompt eradications of infected entities, use of virus-resistant varieties, virus-free certified plants, or chemical prophylaxis against insect vectors can help to contain viral infections (Hull, 2014).

Taking into consideration the serious economic damage caused worldwide by viral diseases, researchers have been committed to introduce genetic resistances against viruses in plants. One of the most promising approaches relied on genetic engineering techniques, an alternative strategy to traditional breeding methods for obtaining virus resistance in several crop species.

One of the key studies in this research area, published by Sanford and Johnston in the mid-1980s, is commonly referred as the Pathogen Derived Resistance (PDR) strategy. The idea behind this concept consists in the ability of plant cells transformed with specific gene sequences derived from the pathogen, to interfere with the replication or the infection of the pathogen itself (Sanford and Johnston, 1985). For plant viruses, the proof of concept of PDR was reported by Abel et al., (1986). In this study, tobacco explants transformed with *Agrobacterium tumefaciens* carrying the coat protein gene (CP) of tobacco mosaic virus (TMV) showed a reduction of virus symptoms when inoculated with TMV. Despite several reports of overexpression of CP or other virus coding sequences, such as replicases, proteinases and movement proteins, the molecular pathways behind this induced resistance were not always clarified (Prins et al., 2008). Later studies revealed that PDR was not always linked to a deregulated synthesis of the corresponding viral proteins, or to the overexpression of dysfunctional viral proteins. Correlations between PDR events and RNA-dependent degradation mechanisms were detected in most cases. This phenomenon was later described as post-transcriptional gene silencing (PTGS) (Lindbo et al., 1993).

2. RNA interference and virus resistance

Between the end of the 1980s and the beginning of the 1990s, two different groups conducting studies on the regulation of gene expression in petunia observed that the overexpression of a foreign sequence homologous to an endogenous plant gene led to specific degradation of both sequences, terming this phenomenon "coordinated suppression" (co-suppression) (Napoli et al., 1990; Van Der Krol et al., 1990). Two years later, a non-translatable CP gene sequence of tobacco etch virus (TEV) was introduced into tobacco plants (Lindbo and Dougherty, 1992). Some of the transgenic lines expressing the TEV-CP gene transcripts developed feeble symptoms when inoculated with TEV, while some of them were symptomless. Surprisingly, the latter presented low steady-state levels of transgenic mRNA,

despite highly active expression. This demonstrated the existence of a cellular-based, sequence-specific, post-transcriptional RNA-degradation system induced by the transgenic mRNA, targeting both the transgene transcript and the homologous virus mRNA for degradation. This was therefore the first described PTGS-based example of virus resistance. Starting from these observations, it has been understood that in plant cells the RNA-mediated virus resistance based on PTGS is part of a natural and complex process now universally known as RNA silencing or RNA interference (RNAi) (Baulcombe, 2004).

The activating molecule of the RNAi machinery is represented by double-stranded RNA (dsRNA) precursors (Voinnet, 2008); in the cytoplasm, DICER-like enzymes identify and specifically cut these dsRNA molecules into small RNAs (sRNAs) composed of 21 - 24 nucleotides (Bernstein et al., 2001; Elbashir et al., 2001; Hamilton and Baulcombe, 1999; Baulcombe, 2004). The sRNAs sense strand, recruited by the RISC complex with the help of Argonaute proteins, is used to scan the cytoplasm in order to find and degrade homologous mRNAs or compromise their translation, thus modulating gene expression (Denli and Hannon, 2003; Ghildiyal and Zamore, 2009; Tijsterman et al., 2002).

Protection against viruses and modulation of endogenous gene expression are the two main field of activity of RNAi in plants (Vazquez et al., 2010). As a gene expression regulator, RNAi functions also in insects (Kennerdell and Carthew, 1998), fungi (Romano and Macino, 1992), animals (Fire et al., 1998) and mammals (Maillard et al., 2019). Moreover, the characteristics of the RNAi mechanism have been exploited to silence invading viral sequences in order to prevent and/or reduce their accumulation in plants. Two main biotech strategies based on the RNAi system have been exploited for crop defence against viruses, known as Host-induced gene silencing (HIGS) and the more recently studied Spray-induced gene silencing (SIGS) method. HIGS depends on the induction of the plant RNAi biological system and is obtained by stable expression of dsRNAs specific for a target virus. As reviewed by Khalid et al. (2017), the activation of PTGS against viruses can depend on the characteristics of the gene constructs introduced in the plant to produce dsRNAs. In this chapter, we offer an overview concerning different hairpin RNA (hpRNA)-based gene construct features and applications, which are definitively considered one of the most powerful tools to induce stable genetic resistance in crops against viruses. On the other side, SIGS, the more recent strategy based on RNAi, relies on the exogenous application of dsRNA molecules homologous to target viral sequences, to trigger the natural RNAi-based defense mechanism towards plant viruses. In this chapter, the major achievements in producing dsRNA molecules in large scale using biofactories and their topical application to plants have been discussed. Moreover, the problems and benefits related to the efficacy and stability of SIGS, compared to HIGS, in particular for field conditions have been discussed.

3. Host-induced gene silencing (HIGS) strategy against viruses: hpRNA silencing approaches

An elegant study published in Nature by Fire et al., (1998) showed that in *Caenorhabditis elegans* RNAi is induced by dsRNA molecules and these molecules were more efficient in inducing silenced phenotypes compared to single-stranded RNA molecules. At the same time, another study demonstrated increased silencing efficiency obtained by the co-expression in the host cell of sense and antisense sequences, compared to their separated expression (Waterhouse et al., 1998).

Later, the expression of dsRNAs was achieved in plants mainly by introducing hairpin RNA (hpRNA) gene constructs, and these were also designed to induce PTGS against viruses. These gene constructs normally include short-inverted sequences homologous to vital viral genes, usually split by a non-coding sequence, such as an intron, all under the control of specific promoters and terminators (Lemgo et al., 2013).

Such a construct strategy was described by Smith et al., (2000) who reported the increase of the silencing effect when an intron-based sequence was inserted as a junction between the sense and antisense arms of the hpRNA construct, leading to almost 100% of independently transformed tobacco lines showing silencing against potato virus Y (PVY). It has been supposed that the intron removal throughout splicing may simplify the folding of the hairpin structure and its transit from the nucleus to the cytoplasm (Wesley et al., 2001). As suggested by molecular analysis carried out on transgenic tomato plants expressing intron hp-RNA-derived sRNAs and resistant to tomato yellow leaf curl virus (TYLCV), it seems that few unspliced hairpin molecules are processed by DCL 3 into 24-nt sRNAs in the nucleus and used as phloem-mobile silencing inducers. On the contrary, spliced hairpin molecules are processed in the cytoplasm by DCL 4 and DCL 2 into 21-nt and 22-nt sRNAs, respectively, and used as cell-autonomous silencing inducers of the target viral sequence (Fuentes et al., 2006, 2016; Pooggin, 2017).

Concerning the choice of the target viral genome sequence selected to build the short-inverted repeats of the hpRNA construct, various aspects have to be considered. All viral genes chosen as RNAi targets for crop defence encode essential proteins necessary for the survival and the replication of the virus in the host, such as coat protein, nuclear capsid protein, replicase and replication associated proteins (Khalid et al., 2017). As recently shown, sequences of different lengths have been chosen and inserted in a wide range of plant species (Cillo and Palukaitis, 2014). In general, essential viral genome portions from 300 up to 800 nucleotides are preferred as target regions (Simón-Mateo and García, 2011), but much smaller sequences (from 23 up to 60 nucleotides) have also been successfully used to induce virus resistance (Thomas et al., 2001). The idea behind such preference in terms of sequence length is connected with the concept that hpRNA-mediated silencing occurs when the homologous region between the hp-derived transcripts and the target viral sequence covers more than 100 nucleotides (Jan et al., 2000; Pang et al., 1997).

The 35S cauliflower mosaic virus (CaMV), the first plant promoter identified almost 40 years ago (Covey et al., 1981), is the most broadly exploited promoter sequence in plant biotechnology also in the case of the hp-RNA constructs design, as it causes constitutively high levels of gene expression in a large variety of plant tissues, despite being derived from a pathogenic virus.

Since the dawn of plant biotechnology, tobacco has been widely exploited as model plant system, mainly to validate the functionality of new gene constructs due to the ease of genetic transformation and virus infection. Since the end of '90s, many achievements and failures in terms of RNA and DNA virus defence via hpRNA have been reported, both in model plants and crops, including several examples where 100% of resistance to the target virus was achieved (reviewed by Khalid et al., 2017). Different hp-gene constructs against several viruses have been evaluated in the model species *Nicotiana tabacum* or *N. benthamiana*, and in twelve cases, ten in *N. benthamiana* and two in *N. tabacum*, respectively, complete resistance was achieved. For example, the production of transgenic *N. benthamiana* plants resistant to citrus tristeza virus (CTV) expressing a hp-gene construct targeting P23+3'UTR sequences led to the application of the same approach in citrus (Batuman et al., 2006). However, following transformation via *Agrobacterium* of the citrus 'Alemow' to enable insertion of a hairpin construct (p23UI), potentially capable of inducing CTV resistance via PTGS, none of the transgenic citrus plants exhibited resistance. This example shows that a result achieved in a model plant may not be directly reproduced in a target crop, possibly since specific host factors participate in the infection process. To partially explain this outcome, it was supposed that a virus could be more virulent in its own natural host than in a different experimental host. To integrate the Khalid et al., (2017) review, it has to be mentioned that the RNAi mechanism has been exploited against plum pox virus (PPV) for the first time by Pandolfini et al., (2003) who designed and introduced an hp-gene construct against PPV in the model species *N. benthamiana*. In this study, a 197 bp-long sequence of the PPV strain D genome was chosen to design the ihprolC-

PP197 gene construct, placed as two inverted repeats separated by a non-coding sequence under the control of the phloem-specific rol C promoter. When the ihprolC-PP197 gene construct has been employed to transform *N. benthamiana* plants, systemic PPV resistance was obtained. Systemic viral infections are common in fruit trees, thus a comparable construct could be developed to achieve PPV resistance also in *Prunus* spp. (Ilardi and Tavazza, 2015).

The RNAi-based strategy was shown to work also against viruses with a DNA genome, as reviewed in (Pooggin, 2017). One of the most intriguing examples of hp-RNA constructs active against the geminivirus TYLCV consisted of the expression of an hp-RNA construct targeting the viral replicase C1 gene (Fuentes et al., 2006). When transgenic lines expressing this construct were tested in field conditions, a long lasting resistance was demonstrated; moreover, the authors highlighted the possibility that this strategy could induce off-target effects and modify the transcriptome of the transgenic lines, as determined by a deep-sequencing approaches (Fuentes et al., 2016; Pooggin, 2017).

4. Transgrafting as a tool to develop genetic resistance against viruses in crops

In worldwide farming, grafting is a very common procedure that basically consists in connecting a portion of a plant (i. e. scion) to another plant (i. e. rootstock), through the junction of their vascular systems. Essentially in a grafted plant system, the rootstock absorbs nutrients from the soil that move to the scion, while the scion synthesizes carbohydrates through photosynthesis that are translocated to the rootstock. The phloem of a grafted organism is where the traffic of plant growth factors, soluble organic compounds, nucleic acids and proteins takes place, creating a dynamic link between rootstock and scion that should lead to an improved growth and yield of the grafted plant (Aloni et al., 2010; Dinant and Suárez-López, 2012; Guelette et al., 2012; Ham et al., 2014). Plant grafting is mostly used for vegetative propagation, to induce resistance against pathogens, to alter plant vigour and increase endurance to abiotic stresses (Aloni et al., 2010; Gonçalves et al., 2006; Koepke and Dhingra, 2013; Kubota et al., 2008).

As explained by Pyott and Molnar, (2015), non-cell autonomous gene silencing signal is "one whose action extends beyond the cell producing the signal". In the late 1990s, the transmission of a silencing signal in the form of dsRNA molecules over long distances was demonstrated by two key studies applied on *N. benthamiana* plants (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). In particular, Voinnet and Baulcombe (1997) induced the stable expression of GFP-encoding sequence in *N. benthamiana* plants and through an optimized *Agrobacterium* infiltration protocol, a temporary GFP silencing was induced in the older leaves of the same treated plants; probably thanks to the translocation of the silencing molecules, a GFP silenced phenotype was detected also in the upper leaves. In the same year, using a grafting procedure, Palauqui et al., (1997) joined wild-type tobacco scions onto transgenic stocks expressing nitrate/nitrite reductase (Nia/Nii) transgene. Chlorosis and reduced amount of Nia/Nii mRNA in the scions suggested a movement of Nia/Nii silencing signals from the transformed stock to the wild-type scion.

The nature of the systemic RNA silencing signal has been an enigma for researchers. At the beginning, it was supposed that the travelling of long dsRNA precursors should take place in the phloem to achieve systemic silencing (Mallory et al., 2001, 2003), but more recent reports suggested that systemic RNA silencing depends almost exclusively on sRNAs as mobile molecules (Buhtz et al., 2008; Chiou et al., 2006; Martin et al., 2009; Melnyk et al., 2011; Molnar et al., 2010; Zhang et al., 2014).

The ability of the silencing molecules to move along the plant vascular system can be exploited in a trans-grafting system (Song et al., 2015). In this case, trans-grafting used as a method to spread sRNAs through the plant and to switch off the replication of a target virus could represent an alternative and promising approach to protect woody plant species against viral diseases. The goal of the hp-RNAi trans-grafting system would be to obtain a cultivar, whose

tissues and organs including pollen and fruits remain untransformed, which is resistant to one or more target viruses thanks to the translocation of sRNAs from an RNAi transgenic grafted rootstock (Lemgo et al., 2013). This approach is particularly suitable to fruit trees species, which are usually propagated vegetatively and not through seeds. For example, peach (*Prunus persica* L. Batsch), grapevine (*Vitis vinifera*; Bouquet et al., 1978) and sweet cherry (*Prunus avium*; Akçay et al., 2008) plants are propagated by grafting to retain the same parental traits in terms of quality and vigour of fruits. Since 1998, transgenic rootstocks have been exploited in fruit woody plants grafting system (reviewed by Song et al., 2015). Two promising examples of virus resistance in non-transgenic scions grafted on transgenic rootstocks were achieved in grapevine (Vigne et al., 2004) and sweet cherry plants (Song et al., 2013; Zhao and Song, 2014); in the latter studies, resistance against prunus necrotic ringspot virus (PNRSV) relies on RNAi mechanism, activated by an hpRNAi-based gene constructs integrated in the grafted transgenic rootstock.

Although the HIGS approach applied to trans-grafting system shows several advantages, especially for inducing plant virus resistance, its use is currently hindered by different issues, especially by the need to generate transgenic plants. Furthermore, this process presents several bottlenecks both from a technical point of view and for regulatory and social aspects (Arpaia et al., 2020; Flachowsky et al., 2009; Fladung et al., 2012; Mezzetti et al., 2020; Taning et al., 2020). In fact, certain crop species are hard to *in vitro* regenerate and/or difficult to genetically transform (Sabbadini et al., 2019). Moreover, the inserted transgenes can be unstable in the host genome or their expression can be silenced or suppressed in the offspring, making transformation ineffective. In addition, the generation and characterization of transgenic lines can be time consuming for some cultivated crops, making unaffordable the evaluation of the effective lines (Altpeter et al., 2016). To reduce or overcome public concerns and bypass technical difficulties to obtain stable and efficient transgenic lines, the exogenous delivery of RNAi effective molecules (sometimes termed Spray-induced gene silencing - SIGS) has been proposed as an appealing alternative for plant disease control (Taning et al., 2020). In this case, the plant host genome is not modified, multi-target strategies are feasible, and the products of this strategy can be obtained in a relatively shorter time (Taning et al., 2020).

5. Spray-induced gene silencing (SIGS) strategy against viruses

The first report of the successful use of exogenously applied dsRNAs against plant viruses dates back to 2001 (Tenllado et al., 2001). In this pioneering work, dsRNA molecules against three different viruses triggered RNA-mediated virus resistance, all with a positive-single stranded RNA genome, such as pepper mild mottle virus (PMMoV), tobacco etch virus (TEV), and alfalfa mosaic virus (AMV). When these viruses were mechanically inoculated on *N. benthamiana* leaves with *in vitro* transcribed dsRNA fragments targeting the PMMoV replicase, the TEV helper component (HcPro) or the AMV RNA3 (fragments of 997 bp, 1483 bp and 1124 bp, respectively), a local antiviral response was elicited, in a dose-dependent manner. However, the authors stated that a certain length of dsRNA was required to reach resistance (Tenllado et al., 2001). Since then, this strategy was applied on many different plant species targeting different viruses, as reviewed in Dalakouras et al., (2020). This work reviews the use of different kinds of formulations of dsRNAs that were delivered on maize plants against sugarcane mosaic virus CP (Gan et al., 2010), on pea against pea seed-borne mosaic virus CP (Šafářová et al., 2014.), as well as on the orchid *Brassolaeliocattleya hybrida* against cymbidium mosaic virus CP (Lau et al., 2014). Other constructs were tested on tobacco targeting the TMV p126 replicase (Konakalla et al., 2016), on cucurbits targeting zucchini yellow mosaic virus HcPro (Kaldis et al., 2018), on *N. benthamiana* targeting a 2611 bp region of the replicase and MP of TMV (Niehl et al., 2018), and on papaya tree against papaya ringspot virus CP (Shen et al., 2014).

For a broad application of dsRNAs in greenhouses and fields, efficient and economically acceptable methods for their large-scale production and purification are required. The initial systems adopted to obtain dsRNAs relies on the *in vitro* enzymatic synthesis of two complementary ssRNA strands, followed by physical annealing (Carbonell et al., 2008.; Tenllado et al., 2001). One of the most frequently used enzymes for ssRNA synthesis is the DNA-dependent RNA polymerases (DdRP) of the bacteriophage T7. For plant virus control, specific target sequences are transcribed by DdRPs from cDNAs template extracted from plants infected by the target virus, using specific primers that carry the T7 promoter at their 5'-end; alternatively the *in vitro* transcription by DdRP can occur starting from plasmids carrying the target viral sequences cloned between two T7 promoters (Konakalla et al., 2016). Different kits are commercially available for this purpose, such as the MEGAscript® RNAi Kit (Life Technologies), Replicator™ RNAi Kit (Finnzymes) or T7 RiboMAXTM Express system (Promega, USA). The production of dsRNAs molecules specifically targeting a selected pathogen region can be followed, optionally, by digestion with DCL enzymes, obtaining a heterogeneous mix of short interfering RNAs (siRNAs), of 18-25 nucleotides in length when the ShortCut® RNase III (NEB, Ipswich, MA) kit is used or of 25–27 nucleotides when the PowerCut Dicer (Thermo Scientific) kit is employed; the siRNA mixture can be further subjected to cleaning with the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA) (Koch et al., 2016; Wang et al., 2016). In a more vigorous *in vitro* system, the ssRNA synthesis performed by the T7 RNA polymerase was coupled to a de novo primer-independent initiation, using the highly processive RNA-dependent RNA polymerase (RdRP) enzyme of bacteriophage ϕ6 (Makeyev et al., 2000), a dsRNA virus infecting *Pseudomonas syringae* cells (Aalto et al., 2007).

To overcome the high costs linked to the *in vitro* dsRNA synthesis, *in vivo* approaches using bacterial cells have been developed, both in *Escherichia coli* (Tenllado et al., 2003; Yin et al., 2009) and in *P. syringae* cells (Aalto et al., 2007; Niehl et al., 2018). In the *E. coli* system, a stably replicating plasmid carrying the target viral sequence cloned within two T7 promoters is introduced into bacteria; following chemical induction of the T7 DdRP gene, that is expressed by a gene cloned in a DE3 prophage or in an additional plasmid, the target sequences are transcribed in both directions; then, the newly generated ssRNA molecules anneal, yielding the desired dsRNAs. Their degradation is inhibited using RNase-III deficient strains, such as E coli HT115 (DE3) or M-JM109lacY, the latter having also a knockout LacY permease gene. This easily scaled-up process is reported to yield about 4 µg dsRNA/ml of bacterial culture (Tenllado et al., 2004).

In a pioneering work, Aalto et al., (2007) described an *in vivo* dsRNA production system in *P. syringae* that have been engineered in order to express the RdRp of bacteriophage ϕ6. This system was further improved using the stable carrier cell line amplifying RNA by means of the phage ϕ6 RdRP (Sun et al., 2004), finally leading to *P. syringae* cells transformed with different plasmids that individually express the viral target sequences, the T7 RdRP, and the ϕ6 RdRP. The dsRNA amplification takes place within the ϕ6 polymerase complexes that also provide a protected environment from bacterial RNases (Niehl et al., 2018; Voloudakis et al., 2015). These bacterial dsRNA production systems can be scaled up, allowing cost-effective large-scale production of long dsRNAs molecules targeting pathogen genes or genomes, suitable for application in crop protection (Niehl et al., 2018).

However, most studies reporting the delivery of dsRNAs produced *in vitro* or *in vivo* showed that the protective antiviral effect lasts only for a few days, indicating insufficient stability or efficacy of these molecules for practical use. As frequent treatments with dsRNAs would be necessary to protect plants from virus infection, especially for long lasting crops cultivated in open fields, establishing methods ameliorating the delivery of dsRNA and their stabilization has become a major challenge. DsRNA formulations based on biocompatible and safe materials are currently being evaluated (Pérez-de-Luque, 2017; Vurro et al., 2019); these include packaging of dsRNAs into virus

particles or in virion-like particles (VLPs) (reviewed in Dalakouras et al., 2020; Zotti et al., 2018). Implementation of dsRNA formulations have been achieved by a biotech company with the 'Apse RNA Containers' (ARCs) system (www.rnagri.com; accessed 17 March 2020). Here, E. coli cells express naturally occurring proteins, such as the CPs from bacteriophage MS2 that can self-assemble and form VLPs. The same cells also contain another plasmid carrying the target RNA precursor signal sequence, linked to a packaging site. During *E. coli* growth, VLPs made of MS2 CP subunits will encapsidate the target RNA molecules.

From another perspective, an elegant breakthrough of the obstacles related to dsRNA delivery relies on the use of non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets of 80-300 nm (BioClay) that bound to dsRNAs and protect them from degradation (Mitter et al., 2017). These BioClay nanostructures are not only resistant to plant watering but also allow gradual release of dsRNAs to the plant cell, leading to more successful inhibition of the propagation of cucumber mosaic virus (Mitter et al., 2017) and bean common mosaic virus in *N. benthamiana* and cowpea (*Vigna unguiculata*) plants, respectively (Worrall et al., 2019).

Other recently developed delivery strategies include direct trunk injection, as in the commercially available Arborject strategy (https://arborjet.com; accessed 17 March 2020) described in Zotti et al., (2018) and Dalakouras et al., (2018), but their efficacy against viruses affecting woody plants remains to be evaluated. Another delivery strategy, which seems to be appropriate for inducing virus resistance in plants, consists in a high-pressure spraying method inducing a symplastic RNA delivery of the effective dsRNA molecules (Dalakouras et al., 2020). Indeed, this technique, first described by Dalakouras et al., (2016), can trigger both local and systemic silencing, and the production of secondary siRNAs, especially when 22-nucleotide are sprayed on the plant tissues.

6. HIGS and SIGS against viruses: biosafety considerations

Although one of the major problems hindering a widespread use of HIGS approach includes the cumbersome regulatory procedures to get governmental approval of transgenic plants, the author would like to highlight twenty-four examples where all the bureaucratic processes reached a fruitful outcome, which are described in detail by Khalid et al., (2017). Among them, a successful case of intron hpRNA-based transgenic common bean plants resistant to bean golden mosaic virus (BGMV) accepted for commercialization in Brazil (Bonfim et al., 2007), which exhibit durable resistance in open fields, with unaltered agronomic characteristics and nutritional value (Aragão et al., 2013; Carvalho et al., 2015). Examples of viral resistant fruit tree species approved for commercial release and generated by HIGS technique, include the papaya ringspot virus (PRSV)-resistant papaya (Fitch et al., 1992), and the PPV-resistant plum (Scorza et al., 2012).

Although, RNAi-based transgenic plants produce only dsRNAs molecules complementary to the target pathogen transcripts, without the synthesis of any new protein, possible off-target effects need to be evaluated. These can be caused by dsRNAs molecules complementarity with unintended sequences in the GM plant or in non-target species (Auer and Frederick, 2009; Frizzi and Huang, 2010; Mlotshwa et al., 2008).

Regarding the use of trans-grafting to obtain RNA-based virus-resistant rootstocks in arboriculture, it is expected that this technology would cause less public concern and that the risk assessment would be limited to the transgenic rootstock, as the scion, fruits and pollen maintain their genetic inheritance. These aspects could encourage, in principle, a simplified approach for their application in agriculture (Lemgo et al., 2013; Petrick et al., 2013). Furthermore, the trans-grafting technology could confer the rootstock characteristics to species or cultivars that have been impossible to genetically transform.

From the biosafety side, the most relevant feature of SIGS relies on the fact that the exogenous application of dsRNA does not involve any modification of the plant genome. Moreover, these substances act by means of their

specific nucleotide sequence, have higher specificity and a reduced tendency to induce pathogen resistance if managed appropriately. Importantly, and contrary to chemical pesticides, dsRNAs are biocompatible and biodegradable compounds, ubiquitously occurring in natural conditions inside and outside organisms (Niehl et al., 2018; Taning et al., 2020). Based on the expert panel of the Toxicology Forum of the 40th Annual Summer Meeting held in 2015, local delivery of dsRNAs is considered safe for human consumption, as RNA molecules are present in all kinds of food and exogenous RNAs are considered free of residues potentially toxic for the plant, food or the environment (Sherman et al., 2015). Nonetheless, to increase the activity and safety of these molecules, careful design and predictions by bioinformatics tools are necessary on a case-by-case basis, in order to avoid off and non-target effects on related or non-related organisms with available genomics information (Zotti et al., 2018).

For the policy relevance of this topic, consensus views on dsRNA-base products have not yet been reached and official legislations governing their use are not yet available in Europe (Mendelsohn et al., 2020). Nonetheless, the European scientific community is currently assessing a regulatory framework for such products, as attested by the Organisation for Co-operation and Economic Development (OECD) Conference on RNAi based pesticides held in April 2019. Noteworthy, the safety and legislation issues for such products are generating heated debates in many countries. For example, in New Zealand, the official Decision of the Environmental Protection Authority considered that exogenous application of dsRNAs was technically out of the area of interest of the legislations on new organisms, and any environmental risk assessments of such kind of products was unnecessary (EPA, 2018); however, this statement generated an active debate with negative reactions in the scientific community (Heinemann, 2019).

7. Conclusions and future prospects

In summary, the major characteristics of HIGS and SIGS strategies so far developed to inhibit the infection and spread of plant viruses have been presented.

HpRNA-mediated HIGS strategy is suitable to target one or more specific viruses by the integration of one or more copies of the transgene in the plant genome (Stoutjesdijk et al., 2002). During the past forty years, the A. tumefaciens T-DNA-mediated gene insertion method has been deeply understood and it is routinely used to transform several plant species, also via HIGS approach. However, some crops are recalcitrant to *Agrobacterium*-mediated transformation, for which alternative transformations strategies may be attempted, such as electroporation, microinjection, or particle bombardment. Despite the fact that HIGS strategy is known to be a durable approach for virus control in agriculture, these plants still suffer from low public acceptance and strict rules for their commercialization and/or release into the environment, especially in the European Union.

The exogenous delivery of dsRNAs to trigger the RNAi mechanism against viruses in plants seems to be a reality for the future of plant disease control, considering that these RNAi effective molecules do not fall under the Directive 2001/18/EC (12 March 2001) of the European Commission or the US regulations, since the plant genome is not modified. In the expectation that regulations of small natural molecules for disease control would include these products as biopesticides, researchers are working to stabilize the formulation of dsRNA molecules suitable for field scale applications at affordable costs.

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THESIS PROBLEM STATEMENT

Following its first detection in Bulgaria in 1932, Sharka disease has been a plague for *Prunus* spp. crops production worldwide (Atanassov, 1932; García and Cambra, 2007). Its etiological agent, plum pox virus (PPV), is included in the Top 10 catastrophic plant virus list (Scholthof et al., 2011) among 1400 plant virus species officially recognized (Lefkowitz et al., 2017). During the last decades, the agronomic impact of Sharka disease has been considerable due to great economic losses. The cost of Sharka management was already esteemed more than 10 billion euros back in 2006 (García and Cambra, 2007). Such economic damage is not only due to the impact of reduced fruit quality, but it takes into account both expensive diagnostic procedures and prevention/containment strategies throught quarantine and eradication of infected trees (García et al., 2014). PPV infection cannot be contained with pesticides because of its easy transmission by aphid vectors in a nonpersistent manner (Harris, 1977). Moreover, the use of conventional breeding techniques to obtain PPV resistance in *Prunus* spp., is mainly limited by their heterozygosity, long reproductive cycle and multiple gene and/or recessive inheritance (Carrasco et al., 2013; Petri et al., 2011). To overcome these limitations, biotechnological approaches exploiting RNAi mechanism are considered promising tools to induce PPV resistance in *Prunus* spp. (Cirilli et al., 2016). Among them, peach (*Prunus persica* L.) is one of the most spread fruit tree species, which counts about 1.712.425 cultivated hectares worldwide reaching the worldwide production of 24.453.425 tonnes in 2018 (FAO STAT, 2018). In addition to its agronomical and economic relevance, peach has been suggested also as a model species for the *Rosaceae* family due to its relatively small genome size (diploid, n = 8), as well as a 2-3 years-long juvenility period, which it is considered to be short in comparison with other fruit tree species (Abbott et al., 2002). However, the stable expression of dsRNAs potentially capable of inducing PPV resistance via PTGS in peach is currently hindered by lack of an efficient genetic transformation protocol suitable for this species. Basically, recalcitrance to *A. tumefaciens* and high rate of chimerism in the few transformed shoots developed are the two main obstacles in obtaining transgenic peach plants. An appealing option to PPV resistance transgenic peaches is represented by the topical application of dsRNAs to peach plants to induce the RNAi pathway against PPV. Nevertheless, the exogenous delivery of RNAi effective molecules in the greenhouse and the field seems arduous, considering that an efficient and low-cost large-scale production, as well as suitable stabilization and delivery of these molecules are required.

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OBJECTIVES

With the final aim to develop strategies to fight Sharka disease in peach, the research program addressed both HIGS and SIGS approaches now used for the application of RNAi technology, including the following activities:

1. **HIGS approach to obtain resistance against PPV in peach:**
   i) Review of the existing literature on peach genetic transformation.
   ii) Development of a protocol for *A. tumefaciens*-mediated transformation, through organogenesis, using meristematic bulks of the peach rootstock Hansen 536 (*P. persica x P. amygdalus*) as starting explants.
   iii) Development of a protocol for inducing organogenesis in the peach rootstock Hansen 536 (*P. persica x P. amygdalus*) using expanding leaves as starting explants.
   iv) Development of protocols for inducing organogenesis and somatic embryogenesis in the peach rootstock GF677 (*P. persica x P. amygdalus*) and in several peach cultivars using different starting explants (expanding leaves, anthers and mature seeds).
   v) Development of protocols for *Agrobacterium*-mediated transformation, through organogenesis, using expanding leaves of the peach rootstock Hansen 536 (*P. persica x P. amygdalus*) as starting explants.

2. **SIGS approach to obtain resistance against PPV in peach:**
   i) Development of a strategy for RNAi molecules production against PPV.
   ii) Development of a strategy for the topical application of RNAi molecules against PPV in the susceptible peach rootstock GF305.

CHAPTER 2

LITERATURE REVIEW

GENETIC TRANSFORMATION IN PEACH (*Prunus persica* L.): CHALLENGES AND WAYS FORWARD[1]

1. Introduction

The genus *Prunus*, belonging to the family *Rosaceae*, includes a large number of fruit tree species known as "stone fruits" because the seed is encased in a hard, lignified stone-like endocarp. The edible portion of the fruit is the fleshy mesocarp, although the genus also includes nut crop species such as almond (*Prunus dulcis* Miller) where the mesocarp development is arrested. The major commercial stone fruit species are apricot (*Prunus armeniaca* L.), European plum (*Prunus domestica* L.), Japanese plum (*Prunus salicina* Lindl.), peach and nectarine (*Prunus persica* L.), sour cherry (*Prunus cerasus* L.), sweet cherry (*Prunus avium* L.), and almond.

Peach has been proposed as a model plant for the *Rosaceae* family (Abbott et al., 2002) due to a relatively short juvenility period (2–3 years) compared to most of other fruit tree species, as well as its genetic characteristics including self-pollination and relatively small genome size (diploid, n=8). In the genus *Prunus*, all constructed linkage maps contain a framework of markers in common with the peach reference physical map "Texas" x "Earlygold" (T x E) (Arús et al., 2012). Furthermore, peach was the first *Prunus* species to be sequenced. The current peach genome version (Peach v2.0) (Verde et al., 2017), generated from a doubled haploid seedling from the cultivar "Lovell", together with the availability of new technologies for high-throughput genome and transcriptome analyses, offers new possibilities for QTL and MTL application and candidate gene identification in all *Prunus* species. Substantial progress has been made in *Prunus* genetics and genomics. The Genome Database for *Rosaceae* (GDR, https://www.rosaceae.org) provides access to all publicly available genomics, genetics, and breeding data in *Rosaceae* (Jung et al., 2018).

Almost 30 years have passed since the first published report on the regeneration of transformed peach plants (Smigocki and Hammerschlag, 1991). Nevertheless, the general applicability of genetic transformation to this species has not yet been established. In the absence of an efficient peach transformation system, progress in determining gene function will remain slow. As an alternative, a highly efficient transformation method in European plum (*P. domestica* L.) has shown to be a useful tool for functional genomics studies in *Prunus* spp. (Petri et al., 2008a). However, peach genetic engineering is not only significant for gene function studies. The lack of efficient peach genetic transformation protocols precludes the application in peach of new biotechnological tools such as RNA interference (RNAi), trans-grafting, cisgenesis/intragenesis, or genome editing in peach breeding programs, as are currently being applied in other fruit tree species (Limera et al., 2017).

Although protocols for plant regeneration from different peach tissues (calli from immature embryos, mature and immature cotyledons, leaf explants) have been reported (e.g., Gentile et al., 2002; Hammerschlag et al., 1985; Mante et al., 1989; Pooler and Scorza, 1995), there are only three reports on regeneration of transgenic peach plants, all from seed-derived tissues (Pérez-Clemente et al., 2004; Prieto, 2011; Smigocki and Hammerschlag, 1991). Unfortunately, none of these reports have been reproduced in other laboratories. Sabbadini et al., (2015) reported the regeneration of two transgenic lines from somatic tissues of the *P. persica x P. amygdalus* hybrid "GF677". More recently, Xu et al., (2020) published an *A. rhizogenes*-mediated transformation method for peach hypocotyl, leaf, and shoot explants to generate transgenic hairy roots to produce composite plants with wild-type shoots and transgenic roots.

Many strategies have been tested in order to obtain an efficient peach transformation system. Despite the amount of time and the efforts invested, the lack of success has meant that much data, potentially useful to the scientific community, has not been published. This review is the result of a collaboration of scientists from different laboratories throughout the world. Here, we present an overview of peach regeneration and transformation research and describe novel strategies and procedures undertaken at our facilities aimed at producing transgenic peaches. Possible future studies and approaches are discussed.

2. State of the art work in peach transformation

The development of a system for gene transfer or gene editing in peach depends upon the availability of effective regeneration procedures coupled with techniques that permit efficient DNA delivery, selection of transformed tissues, and recovery of transgenic plants. Unfortunately, *P. persica* is universally known to be one of the most recalcitrant species in terms of production of transformed plants (Prieto, 2011). Table 1 summarizes the results published to date on peach genetic transformation. To the best of our knowledge, other than these published results, Okanagan Specialty Fruits (OSF) Inc. (Summerland, BC, Canada) achieved success in the 2000s through developing some peach transformed lines with a procedure that involved somatic embryogenesis (SE). However, the effciency of the technique was very low, and their success was based on the extremely high number of explants used. Currently, the company has abandoned this line of research (John Armstrong, personal communication).

Table 1. Transformation in *Prunus persica* L.

Genotype	Method (strain)	Plasmid (genes)	Explant	T.E.[a] (%)	Main advantage	Main disadvantage	Reference
'14DR60'	*A. tumefaciens* (A281)	pGA472 (*npt*II)	Embryogenic callus, leaves and immature embryos	0	All three starting explants developed calli which were able to grow in a medium containing the selective agents.	Typically, long term embryogenic peach cultures produce few normal shoots.	Scorza et al. (1990)
'Tennessee natural'							
'PER 2D'							
'Redhaven'	*A. tumefaciens* (tms328::Tn5)	pTiA6 (*iaa*, *ipt*)	Shoots	0	Demonstration of potential for using *A. tumefaciens* to transfer genes to peach.	Shoots could not be regenerated from the transformed cells.	Hammerschlag et al. (1989)
			Immature embryo axes	n.s.	Demonstration of regeneration of plants from embryo-derived callus infected with the shooty mutant strain of *A. tumefaciens*.	Not reproduced in other laboratories.	Smigocki and Hammerschlag (1991)
'Lovell'	Biolistic	pBI505, pBI426 (*npt*II, *gus*)	Embryo calli, immature embryos, cotyledons, leaves and shoot tips	0	Optimization of biolistic parameters for this species.	Unsuccessful recovery of plants from the transformed embryogenic calli.	Ye et al. (1994)
'Miraflores'	*A. tumefaciens* (C58C1/pMP90)	pBin19-sgfp (*npt*II, *gfp)*	Mature embryo axes	3.6	Mature tissue available year-round.	Not reproduced in other laboratories.	Perez-Clemente et al. (2004)
'Bailey'	*A. tumefaciens* (LBA4404, EHA105, GV3101, CG937, CG1052, CG1059)	pLC101 (*npt*II, *gfp)*	Cotyledons, embryonic axis, hypocotyl slices, callus, internodes and leaves	0	Comprehensive evaluation of factors affecting *A. tumefaciens*-mediated peach transformation. Seed-derived internodes showed the highest transformation percentage compared to the other explants.	Rates of GFP transformation under the experimental conditions were low.	Padilla et al. (2006)
'Lady Nancy'							
'Harrow Beauty'							
'KV930465'	*A. tumefaciens* (LBA4404, EHA105)	pBin19 (*npt*II, *gus)*					
'KV930408'							
'KV930303'							
'KV939455'	*A. tumefaciens* (LBA4404)	pBISNI, pGA482Ggi (*npt*II, *gus)*					
'KV930478'							
'KV930311'							
'Akatsuki'	Electroporation	pBI221, pE2113-GUS, PL-GUS (*gus*)	Protoplasts from immature fruits mesocarp	0	The system can be applied for expression analysis of genes isolated from other *Rosaceae* species.	The period suitable for protoplast isolation is limited to about 1 week.	Honda and Moriguchi (2006)
'O'Henry'	*A. tumefaciens* (GV3101, EHA105)	pBIN-m-gfp5-ER (*npt*II, *gfp*)	Immature cotyledons	0.6	Very efficient regeneration protocol.	Explants available for only a limited time each year (50 to 70 days post bloom). Not reproduced in other laboratories.	Prieto (2011)
'Rich Lady'							
'GF677'[b]	*A. tumefaciens* (GV2206)	hp-pBin19 (*npt*II)	Meristematic bulks	0.3	The first successful report of a peach rootstock genetic transformation using adult tissue as starting material.	The efficiency of the procedure was relatively poor.	Sabbadini et al. (2015)
'Hansen 536'[b]	*A. tumefaciens* (EHA105)	pK7WG2-ihp35S-PPV194::eGFP (*npt*II, *gfp*, *PPV polyprotein*	Meristematic bulks	0	Uses adult tissues as source of explants.	Shoot regeneration from transgenic calli was not obtained.	Sabbadini et al. (2019)

		hairpin)					
	A. tumefaciens (EHA105, LBA4404, GV3101)	pBISN1 (*npt*II, *gus*)	Leaves	0	Adult tissue available year-round.	Only transient transformation was recorded.	Zong et al. (2019)
'Shantao'	*A. rhizogenes* (MSU440)	pMV2G + Ri Plasmid (*DsRED1*) + (*rol* genes)	Leaves, hypocotyls and shoots	27.8[c]	This protocol provides a way to evaluate gene functions, genetic engineering, and root-rhizosphere microorganism interaction in peach.	Only transgenic hairy roots were regenerated. Transgenic shoots were not produced.	Xu et al. (2020)
'Shengli'				50.9[c]			
'Lvhuajiuhao'				30.7[c]			
'Shengli'		pSAK277 (*PpMYB10.1*)	Shoots	n.s.[c]			

[a] Transformation efficiency (number of transgenic shoots obtained per 100 explants). When non indicated, it was not specified (n.s.) by authors. [b] *P. persica x P. amygdalus* hybrids. [c] Efficiency of regeneration of transgenic hairy roots.

2.1. Type of explant

There are two classes of explants that may be used for regeneration of transformed plants: juvenile material (seed-derived tissues) or adult material. Regeneration from adult somatic tissues is highly recommended for clonally propagated crops in order to maintain genetic uniformity of the cloned plants, especially for the highly heterozygotic *Prunus* species. A procedure that allows the genetic transformation of a range of clonally propagated genotypes would be the ideal situation, not only for peach but for any woody fruit species. Unfortunately, procedures that use clonal tissues as the source of explants cannot be readily transferred among genotypes. Several reports (Gentile et al., 2002; San et al., 2014; Zhou et al., 2010; Zong et al., 2019) showed the diffculty in establishing a standard protocol for peach leaf organogenesis. Typically, these protocols are highly genotype-dependent and are influenced by the combination of factors such as the type and age of starting donor explant, basal medium composition, dark/light period during culture, and plant growth regulators supplemented to basal culture medium. Despite these above-mentioned regeneration studies, there are no routine genetic transformation systems reported for any peach genotype.

Currently, most of the transformation procedures in *Prunus* spp. involve the use of seed-derived explants, including apricot (Laimer da Câmara Machado et al., 1992; Petri et al., 2015), European plum (Petri et al., 2008; Tian et al., 2009; Wang et al., 2013) and Japanese plum (Urtubia et al., 2008). While transformation of peach from seed explants has been reported (Pérez-Clemente et al., 2004; Prieto, 2011; Smigocki and Hammerschlag, 1991) the successes have not been repeated in other laboratories. If a routine transformation method for peach seed-derived tissues were to be developed, it could have an impact on the development of new rootstock varieties, and it would also allow for the introduction of novel genes into the peach germplasm that could be used in conventional breeding programs, especially in view of the relatively short generation time for peach.

2.2. DNA delivery method

Ye et al., (1994) optimized biolistic parameters for peach. Bombardment was applied to different tissues, but transformation was stable only in the zygotic embryo-derived calli. They obtained 65 putative transformed calli lines, 19 of these produced shoot-like structures, but shoots were not recovered.

Several studies have investigated factors affecting *Agrobacterium*-mediated gene transfer in peach. Different peach tissues, such as embryogenic calli, leaves, and immature embryos, are amenable to *A. tumefaciens*-mediated transformation (Scorza et al., 1990). *Agrobacterium*-mediated transformation of multiple types of explants from different genotypes using diverse bacteria strains harboring different plasmids have been evaluated (Padilla et al., 2006). The combinations utilized had a strong influence in the percentage of infected explants expressing the reporter genes green fluorescent protein (gfp) or β-glucuronidase (gus), suggesting that it will be necessary to adjust strain/plasmid/promoter/vector with each type of explant to optimize transformation and regeneration effciencies. In that study, seed-derived internodes showed the highest transformation percentages of 56.8% and 26.0% on the basis of GUS or GFP detection, respectively, compared to other explants such as cotyledons, leaves, or embryonic axes (Padilla et al., 2006). Zong et al., (2019) found the strain EHA105 as the most effcient for transient transformation in the peach-almond hybrid rootstock “Hansen 536” leaves, compared to GV3101 and LBA4404.

Zimmerman and Scorza (1996) reported on the success of a procedure combining biolistic and *A. tumefaciens* for the transformation of tobacco meristems and the production of transgenic plants. However, when tested on peach, they encountered a significant mortality rate due to the mechanical damage and desiccation during dissection to expose the meristems. In addition, bacterial growth was diffcult to control (Scorza et al., 1995).

2.3. Transgenic peach plant recovery

As stated previously, currently there are only three publications reporting the regeneration of transgenic *P. persica* plants (Pérez-Clemente et al., 2004; Prieto, 2011; Smigocki and Hammerschlag, 1991). The first report utilized a "shooty mutant" strain of *A. tumefaciens*. This strain carried a Ti plasmid with a functional isopentenyl phosphotransferase gene (ipt), involved in cytokinin biosynthesis, and a Tn5 transposon-inactivated auxin biosynthesis gene (iaaM). The infection with a "shooty mutant" strain induces the development of tumors, from which transgenic shoots regenerate. Peach tissues transformed with the ipt gene allowed selection of transformed shoots on a medium low in plant growth regulators (PGRs). *In vitro* assays of these plants demonstrated delayed senescence on cytokinin-free medium as compared with non-transformed controls. The resulting peach plants were shorter in stature than controls, and one line exhibited greater branching, presumably due to the effect of the ipt transgene expression (Hammerschlag and Smigocki, 1998).

Pérez-Clemente et al. (2004) using longitudinal mature embryo slices as the explant source, reported the regeneration of transgenic plants expressing the nptII, which confers resistance to aminoglycoside antibiotics and gfp marker genes with a transformation effciency of 3.6 ± 1.0 %. This protocol improved upon the preceding report of Smigocki and Hammerschlag (1991) in that mature embryos are available year-round while immature embryos are available for only a limited time each year.

The most recent report relies on a procedure using SE from immature peach cotyledons. It describes the production of transformed plants expressing gfp from "O'Henry" and "Rich Lady" immature cotyledons with a transformation effciency of about 0.6% (Prieto, 2011).

Sabbadini et al. (2015) reported an *A. tumefaciens*-mediated transformation protocol using "GF677" (*P. persica x P. amygdalus*) meristematic bulk (MB) slices as starting material. Meristematic bulks (MBs), initiated from shoot tips, mechanically and chemically treated, differentiated, and regenerated adventitious shoots. After 32 weeks of selection with kanamycin, the efficiency of the procedure was relatively poor (0.3%), and when this methodology was applied to "Hansen 536", another peach x almond hybrid rootstock, only produced transgenic callus lines (Sabbadini et al., 2019).

2.4. Methodologies for functional genomics studies

Honda and Moriguchi (2006) described a protocol for transient gene expression analysis using protoplasts isolated from immature peach fruits. Xu et al., (2020) developed an *A. rhizogenes*-mediated transformation method to generate transgenic hairy roots from peach shoots to produce composite peach plants with transgenic roots and non-transgenic shoots. They proposed this method for studying root–rhizosphere microorganism interactions in peach and as a method for clonal propagation. The authors also demonstrated the applicability of the system to assess endogenous gene functions. Regarding methodologies for functional genomics studies in peach, it is interesting to note the induction of RNA silencing through a prunus necrotic ringspot virus (PNRSV) viral vector for virus-induced gene silencing (VIGS) (Cui and Wang, 2017). They demonstrated that the PNRSV-based vector could effciently silence endogenous genes in peach.

3. Further approaches applied to improve peach regeneration-transformation

This section summarizes the results of different strategies/procedures aimed at producing transgenic peaches performed at our facilities (Table 2). Although we are working in laboratories throughout the world, we have collaborated in the past and continue active cooperation in order to obtain an effcient peach transformation system. Our

approaches have thus far failed at developing a robust peach transformation protocol. Nevertheless, our findings represent incremental progress towards this goal and are potentially useful to the scientific community. In the subsequent discussion, the work of this group is presented without identifying individual collaborators.

Table 2. Further approaches applied to improve *P. persica L.* regeneration-transformation

Genotype	Explant	Route of morphogenesis	Shoot regeneration rate[a] (%)	Transformation method	Plasmid (genes)	Selection strategy (agent)	Outcome/ Comments	Main advantage	Main disadvantage
Assessed methodologies that involve peach juvenile tissues									
'O'Henry', 'Elegant Lady', 'Rich Lady', 'Venus'	Immature cotyledon	Somatic embryogenesis	60.0	Not assayed	--	--	A similar regeneration procedure couple with *A. tumefaciens* has previously succeed in the generation of transgenic peach plants (Prieto 2011).	Consistent whole plant production.	Explants available for only a limited time each year (50 to 70 days post bloom).
'Bailey', 'Guardian', 'Starlite'		Organogenesis	80.0	*A. tumefaciens* GV3101	pVNFbin (*nptII, gus*)	Negative: Early or late (kanamycin)	Selection failed. All surviving shoots were escapes.	Efficient adventitious regeneration.	Explants available for only a limited time each year (45 to 50 days post bloom).
'TruGold'	Mature hypocotyl slice	Organogenesis	14.0	*A. tumefaciens* (EHA101, GV3101)	pVNFbin (*nptII, gus*)	Negative: Early (paromomycin)	Selection failed. All surviving shoots were escapes.	Explants available year-round (mature seeds can be stored at 4 °C for several years).	Plants regenerated from transformed tissues via organogenesis may be chimeras.
'Bailey'			32.0	*A. tumefaciens* EHA105	*ipt*-containing construct (*ipt, gus*)	Positive: Early (Low level TDZ)	Selection failed. All regenerated shoots were escapes.		
'Nemaguard'			43.0	*A. tumefaciens* EHA105	pBarGUS (*bar, gus*)	Negative: Early (BASTA)	Selection failed. All surviving shoots were escapes.		
'Bailey'	Seed-derived internode	Organogenesis	42.9	*A. tumefaciens* EHA101	pVNFbin (*nptII, gus*)	Positive: Early (Paromomycin)	All shoots died during selection.	Explants available year-round (mature seeds can be stored at 4 °C for several years).	Plants regenerated from transformed tissues via organogenesis may be chimeras.
Assessed methodologies that involve peach adult tissues (cultivars or rootstocks)									
'Hansen 536' [b]	Leaf from meristematic bulk	Somatic embryogenesis	0	Not assayed	--	--	--	Explants available year-round.	Potential for somaclonal variation due to the use of a highly differentiated tissue such as leaf.
'GF677' [b]	Petal and anther		0			--		Regeneration via SE reduces the formation of chimeras.	Explants must be tested at different developmental stages, which can influence SE.
'EVD 1', 'EVD 2', 'EVD 3', 'EVD 44288',	Leaf	Organogenesis	0 (only root regeneration	Not assayed	--	--	--	Explants available year-round.	Inefficient adventitious shoot

'Redglobe', 'Redhaven', 'Coacalco-OP[c]', 'Rutgers Redleaf-OP', 'Sihung Chui Mi-OP', 'Nemaguard-OP', 'Indian Cling OP'			observed)						regeneration protocol.
'Bailey-OP'	Axillary shoot	Organogenesis	100.0	*A. tumefaciens* (GV3101, EHA105)	pSGN (*nptII, eGFP*)	Negative: Early (Kanamycin)	Selection failed. All surviving shoots were escapes.	Consistent whole plant production.	Plants regenerated from transformed tissues via organogenesis may be chimeras.
	Nodal explant	Organogenesis	73.3	*A. tumefaciens* EHA101	pVNFbin (*nptII, gus*)	Negative: Early (Paromomycin)	Two chimerical shoot lines detected (1.7% transformation efficiency).	Explants available year-round.	
'UFO-3', 'Alice Bigi', 'Garnem'[b]	Meristematic bulk	Organogenesis	25.0, 8.3, 91.6 (respectively)	*A. tumefaciens* (C58, EHA105)	pBin19-sgfp (*nptII, GFP*)	Negative Early (Kanamycin)	Transient transformation	The explants are produced in a relatively short period of time (90 days).	Certain probability of somaclonal variation induced by increasing concentrations of cytokinins applied to the initial explant.
'Hansen 536'[b]			80	*A. tumefaciens* EHA105	pK7WG2-ihp35S-PPV194::eGFP (*npt*II, *gfp*, *PPV polyprotein* hairpin)	Negative: early or late (Kanamycin)	Stably transformed calli		

[a] Shoot regeneration rate under non-selective conditions. [b] *P. persica x P. amygdalus* hybrids. [c] OP: Open-pollinated. [d] Transformation experiments were not performed in this case.

3.1. Assessed methodologies that involve peach juvenile tissues

3.1.1. SE from juvenile tissues

The culture of seed-derived explants can be considered as a first stage in the protocol leading to SE. Seed maturity stage and genotype affect the induction and/or the development of the organized structures (Druart, 1999; Scorza et al., 1990b; Srinivasan and Scorza, 2007). SE has been initiated from friable callus (Hammerschlag et al., 1985), longitudinal cotyledonary slices (Bhansali et al., 1990), and cultured immature zygotic embryos (Srinivasan and Scorza, 2007). We have observed that immature cotyledons (50 to 70 days post-bloom) cultured in LP medium (Gentile et al., 2002) and supplemented with 6-benzylaminopurine (BAP; 5.0 μM) and α-naphthaleneacetic acid (NAA; 3.0 to 5.0 μM) led to consistent SE production in "O'Henry", "Elegant Lady", "Rich Lady", and "Venus" peaches. Although genetic transformation was not evaluated, this procedure (Figure S1) allowed whole-plant production in the above-mentioned genotypes.

3.1.2. Organogenesis from juvenile tissues

Immature cotyledons

A regeneration protocol for "Bailey", "Guardian", and "Starlite" immature cotyledons produced around 80% regeneration (Figure 1 and Procedure S1). The addition of 60 μM silver thiosulphate (STS) to the regeneration media and a two-step strategy to recover the buds allowed the successful regeneration, development, elongation, and further establishment of adventitious shoots in a greenhouse (Figure 1), significantly improving the results compared to those previously reported (Srinivasan and Scorza, 2007).

This regeneration protocol (Procedure S1) was combined with *Agrobacterium*-mediated transformation and two different selection strategies (Figure 1f): an early selection, applying 10 mg/L kanamycin right after the co-cultivation, and a late selection, where kanamycin (10 mg/L) was applied at the elongation stage. Immature cotyledons of "Starlite", "Bailey", and "Guardian" were infected with *A. tumefaciens* GV3101 harboring the pVNFbin binary plasmid (Wang et al., 2001). On the basis of PCR analysis with specific primers for the *gus* gene, a total of 21 putative transgenic shoots were obtained. Only a few clones survived the entire selection procedure and were transferred to a greenhouse. Subsequent molecular tests (Southern blot analysis) showed that all were escapes (not shown). These results suggested that neither selection strategy allowed the survival of non-transformed or chimerical plants. In future studies, the application of a gradual increasing selection strategy might eliminate the recovery of chimeric plants and non-transformed escapes.

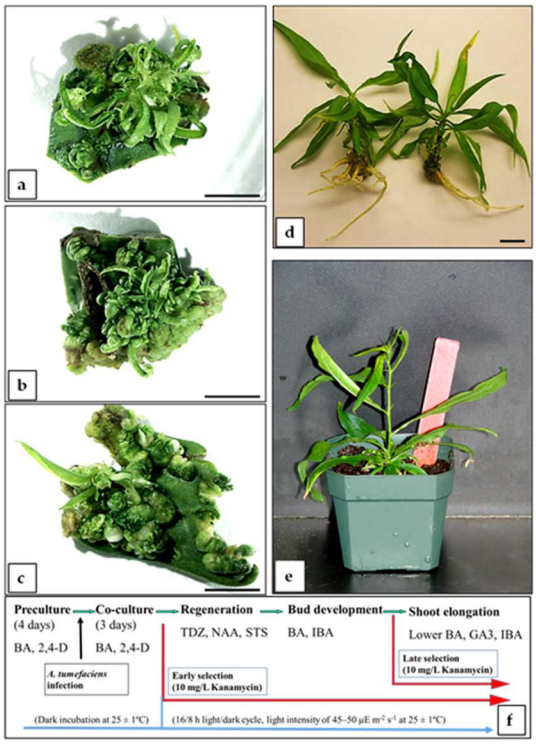

Figure 1. Direct adventitious regeneration from immature peach cotyledons: **(a–c)** Bud regeneration observed in immature cotyledons of "Starlite", "Bailey", and "Guardian", respectively, under no selection regime and controls after 5–6 weeks from the beginning of the experiment (bar = 0.5 cm). **(d)** Rooted shoots after 4 weeks in rooting medium prepared for acclimatization (bar = 1 cm). **(e)** Potted plant cultured in a greenhouse after the rooting and acclimatization process. **(f)** Scheme of the methodology followed for regeneration of transformed shoots.

Mature seed hypocotyl slices

Different factors affecting adventitious regeneration from hypocotyl slices were studied such as basal media; gelling agents; different types, concentrations, and combinations of PGRs; 2, 4-Dichlorophenoxyacetic acid (2, 4-D) pulses; dark induction periods; addition of ethylene inhibitors, such as STS or 2-aminoethoxyvinyl glycine (AVG); polyamines; and coconut water.

Adventitious buds were observed as direct organogenesis after 4 weeks of culture from the beginning of the experiment, and additional buds appeared in the subsequent 2–3 weeks (Figure 2 a, b). Most of the factors studied did not increase or affect adventitious regeneration. Results showed that QL basal salts (Quoirin and Lepoivre, 1977) slightly increased regeneration rates compared to MS salts (Murashige and Skoog, 1962) (data not shown). The effect of the ethylene inhibitors (STS and AVG) was genotype-dependent (Figure 2 c, d). A synergistic effect was not found when both ethylene inhibitors were added to the regeneration medium (data not shown). A dark induction period appeared to be important in peach organogenesis from mature hypocotyl explants. One or two weeks in the dark significantly increased ($p < 0.01$) the regeneration rate for both of the cultivars tested, "Nemaguard" and "Bell of Georgia" (Figure 2 e). Because of this study, the most appropriate conditions for adventitious regeneration from peach mature seed hypocotyl explants were determined (Procedure S2). Regeneration rates were 32% for "Bailey", 28% for "Bell of Georgia", 34% for "Bounty", 33% for "Lovell", 43% for "Nemaguard", and 14% for "TruGold".

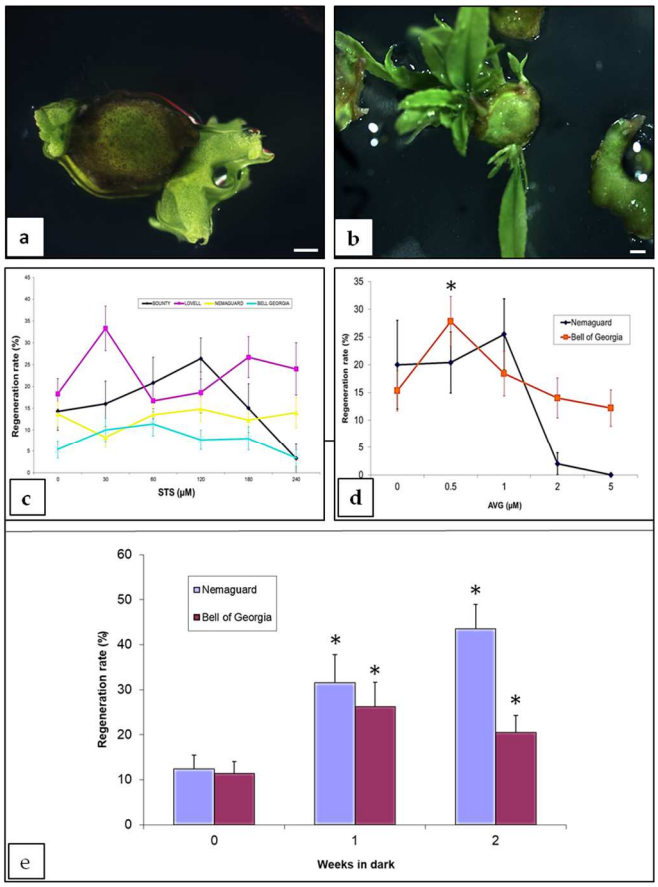

Figure 2. Factors affecting adventitious regeneration from peach mature seed hypocotyl slices. (**a**) First buds appearing after 4 weeks of culture from the beginning of the experiment (bar = 1 mm). (**b**) Adventitious regeneration from a peach hypocotyl section after 6 weeks of culture from the beginning of the experiment (bar = 1 mm). (**c**) Effect of silver thiosulphate (STS) on regeneration. A total of 182, 498, 862, and 700 explants were used in this experiment for "Bounty", "Lovell", "Nemaguard", and "Bell of Georgia", respectively. Vertical bars indicate standard errors (SE). (**d**) Effect of 2-aminoethoxyvinylglycine (AVG) on regeneration. A total of 229 and 484 explants were used in this study for "Nemaguard" and "Bell of Georgia", respectively. Vertical bars indicate SE. Asterisks indicate significant regeneration increased ($p < 0.01$) compared to the control without addition of AVG, according to Pearson's chi-test. (**e**) Effect of dark incubation period on regeneration. A total of 255 and 326 explants were used in this study for "Nemaguard" and "Bell of Georgia", respectively. Vertical bars indicate SE. Asterisks indicate statistical significance ($p < 0.01$) compared to the treatment without dark induction, according to Pearson's chi-test. All the experiments were repeated at least twice.

Following this regeneration method (Procedure S2), two different selection strategies were considered: (i) an aminoglycoside antibiotic-based selection strategy, or (ii) selection with the herbicide BASTA. Experiments to establish the inhibitory concentrations of the selective agents for the different peach cultivars were performed, and regeneration inhibition curves for aminoglycoside antibiotics (kanamycin and paromomycin) and BASTA herbicide were established (Figures 3 and 4). A total of 10 mg/L of kanamycin or 40 mg/L of paromomycin were necessary to inhibit regeneration (Figure 3 a). When paromomycin was added to the media, explants looked healthier than in the presence of kanamycin (Figure 3 b). Peach hypocotyl sections appeared very sensitive to the herbicide BASTA. Regeneration inhibitory concentrations varied among the genotypes tested, being 0.5 mg/L for "Bell of Georgia" and "Bounty", 1.0 mg/L for "Nemaguard", and 2.5 mg/L for "Lovell" (Figure 4). The two highest concentrations tested (2.5 and 5.0 mg/L) were highly toxic, and all explants exposed to them died (Figure 4).

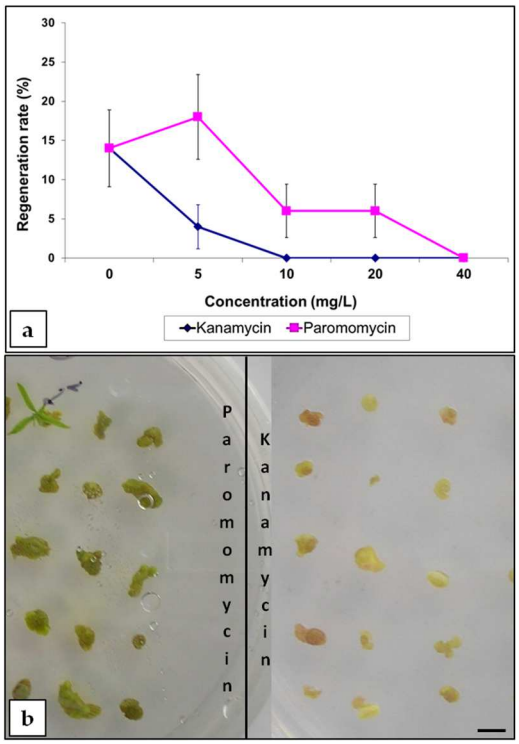

Figure 3. Effect of aminoglycoside antibiotics (paromomycin and kanamycin) on mature peach seed hypocotyl sections. (**a**) Effect on adventitious bud regeneration. For this study, 450 explants were used (cv. "Bell of Georgia"). The experiment was repeated at least twice. Bars indicate SE. (**b**) Explants incubated in regeneration medium containing 20 mg/L of the specified antibiotic after 5 weeks of culture (bar = 0.5 cm).

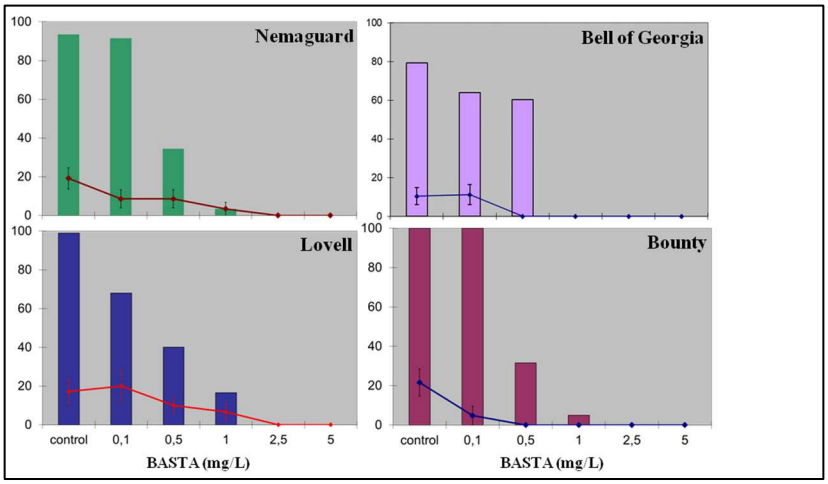

Figure 4. Effect of BASTA herbicide on mature peach seed hypocotyl sections. Color column charts represent explant survival (%) after 2 weeks from the beginning of the experiment. Line charts represent regeneration rates (%) after 7 weeks from the beginning of the experiment with the vertical bars indicating SE. A total of 177, 264, 132, and 183 explants were used in this experiment for "Nemaguard", "Bell of Georgia", "Lovell", and "Bounty", respectively. Experiments were repeated at least twice.

In other woody species, such as apple and apricot, it has been suggested that substantial necrosis in non-transformed tissues under selection pressure could inhibit regeneration from transformed cells (Petri et al., 2008; Szankowski et al., 2003). As illustrated in Figure 3 b, non-transformed hypocotyl sections cultured in the presence of paromomycin remained green, suggesting that this could be a more appropriate selective antibiotic than kanamycin for this peach explant. Following a similar strategy, 0.1 – 0.5 mg/L of BASTA was established as the proper selective concentration (depending on the genotype), severely reducing regeneration but allowing explant survival.

A set of experiments was carried out, with the *A. tumefaciens* strains EHA101 and GV3101 harboring the pVNFbin binary plasmid (Wang et al., 2001) containing the selective marker gene *npt*II. Another set of experiments was performed with the *A. tumefaciens* strain EHA105 harboring the pBarGUS plasmid (Fromm et al., 1990), the *bar* selective marker gene conferring resistance to BASTA herbicide. Both plasmids contained an intron-containing *gus* gene, which prevents expression of the gene by bacteria, as the transformation reporter gene. After 3 days of co-cultivation, infected hypocotyl explants (cv. "Bailey" and "TruGold") were placed in selective medium containing 40 mg/L paromomycin (*npt*II-transformed tissues) or 0.1 mg/L BASTA (cv. "Nemaguard") (bar-transformed tissues). Stable transformation was evaluated with histochemical gus assays (Jefferson, 1988) after 7 weeks from the beginning of the experiments. There were low transformation rates for both constructs used. On average, 20% of the infected explants showed only a few GUS spots on their surface. Regeneration rates in the infected explants were similar to those observed in the non-transformed explants (Figures 3a and 4), and transgenic "blue" shoot buds were not observed. This work indicated that negative selection strategy was not appropriate.

In addition, a positive selection strategy was tested. In this case, selection was not based on toxicity for non-transformed tissues. Using a positive selection strategy, transformed cells have an advantage over non-transformed cells, allowing them to proliferate and differentiate into new adventitious buds. Following an approach similar to Smigocki and Hammerschlag (1991), *ipt* was used as the selective marker gene. Hence, only *ipt*-transformed cells should be able to regenerate in a cytokinin-free or low-level regeneration medium.

Following the regeneration procedure described above (Procedure S2), we compared the effect of the thidiazuron (TDZ) concentration on the organogenesis of "Bailey" hypocotyl sections among non-infected and infected explants using the *A. tumefaciens* EHA105 strain harboring the *ipt*-containing construct. The effect of the *ipt* gene was evident on adventitious root regeneration (Table 3). The frequency of root regeneration was clearly reduced on infected explants compared to the non-infected controls (Table 3) indicating that the IPT enzyme increased the cytokinin to auxin ratio. However, a marked effect of ipt on shoot regeneration relative to controls was not observed for any of the TDZ concentrations tested (Table 3). A total of 65 putative transgenic buds were isolated and elongated. Molecular analyses (PCR and/or DNA blot) revealed that all of them were escapes.

Table 3. Effect of thidiazuron (TDZ) concentration on peach (cv. "Bailey") mature seed hypocotyl section organogenesis: comparison among non-infected and infected explants with *A. tumefaciens* EHA105 strain harboring an ipt-containing construct.

TDZ (µM)	Treatment	Explants	Shoot regeneration (%)	Root regeneration (%)
0	control	70	2.9	85.7
	ipt-infected	39	7.7	38.5
2.5	control	80	10.0	20.0
	ipt-infected	65	7.7	7.7
5.0	control	104	21.1	26.0
	ipt-infected	350	12.3	2.3
7.5	control	74	12.2	8.1
	ipt-infected	79	15.2	3.8

Experiments were repeated at least twice.

Seed-derived internodes

Following the study of Padilla et al. (2006), where seed-derived internodes showed the highest *Agrobacterium*-mediated transformation rate, we examined the organogenic potential of these explants. Histological studies demonstrated the absence of preformed buds or shoot primordia in the explants at day 0 (Figure 5 a), whereas at day 9, meristematic domes with leaflets were observed as emerging from the epidermis of the internode (Figure 5 b). The best regeneration rates (42.9%) for "Bailey" explants were reached when 10.0 µM BAP was added to the medium and seeds

were germinated in the presence of 40.0 µM BAP (Table 4). Shoots regenerated from the central part of the explant (Figure 5 c), coinciding with the area of greater GUS and GFP expression (Figure 5 e, f). Following this regeneration protocol (Procedure S3), we carried out *Agrobacterium*-mediated transformation experiments with the EHA101 disarmed strain containing the pVNFbin binary plasmid. Selection with 20 mg/L paromomycin was applied right after a 2-day co-cultivation. Paromomycin at 20 mg/L inhibited regeneration from non-infected explants. On the other hand, 12.2% of the infected explants showed shoot regeneration (Figure 5 d) and around 30% of the assayed explants showed few GUS spots. Transgenic shoots were not obtained since all regenerated buds died during selection.

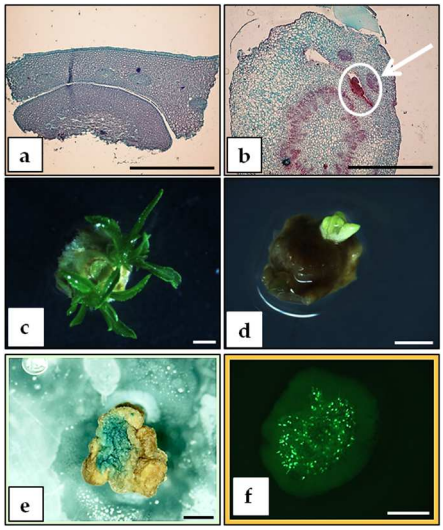

Figure 5. Regeneration and transformation frompeach seed-derived internode explants. (**a**) Histological study of the internode/cotyledon attachment area at day 0 (bar = 1 mm). (**b**) Histological study of the internode/cotyledon attachment area at day 9. Internode with axillary bud with evident meristematic dome with leaflets growing from the epidermis (arrow) (bar = 1 mm). (**c**) Adventitious shoot regeneration from a peach internode explant (bar = 1 mm). (**d**) Adventitious shoot regeneration from a EHA101 pVNFbin-infected explant cultured in regeneration medium supplemented with 20 mg/L paromomycin (bar = 1 mm). (**e**) β-Glucuronidase (GUS) activity in a peach internode explant (bar = 1 mm). (**f**) Green fluorescent protein (GFP) activity in a peach internode explant (bar = 1 mm).

Table 4. Organogenesis from germinated peach seeds internodes (cv. 'Bailey')

Citokinin added to the regeneration medium	**Seed germination**					
	without BAP			**40 µM BAP**		
BAP (µM)	**Regeneration (%)**	**Shoots/explant**	**Roots (%)**	**Regeneration (%)**	**Shoots/explant**	**Roots (%)**
0	3.7 ^{c}	1	0	0 ^{c}	0	40
1	10 ^{bc}	1.3	0	0 ^{c}	0	18
5	30.6 ^{a}	1	0	19.1 ^{b}	1	0
10	39.3 ^{a}	1.7	0	42.9 ^{a}	1.7	0

Data were collected after 8 weeks from the beginning of the experiment. Experiment was repeated three times with 10 explants per treatment. Different letters indicate statistical significance ($p < 0.05$) according to chi-square test.

3.2. Assessed methodologies that involve peach adult tissues (cultivars or rootstocks)

3.2.1. SE from peach adult clonal material

To the best of our knowledge, somatic embryos from peach mature tissues have not been obtained to date. The aim of the study described here was to obtain a protocol for SE from different peach mature tissues.

SE from leaf explants

Young leaves from *in vitro* meristematic bulks (MBs) of the commercial peach rootstock "Hansen 536" (*P. persica x P. amygdalus*), obtained following the protocol described by Sabbadini et al. (2019), were used as starting

explants (Figure 6 a). Leaves were cultured with the abaxial side in contact with the SE induction media (Procedure S4), supplemented with several combinations of PGRs and aminoacids (Table SM1). In these experiments, there was no SE induction from "Hansen 536" on any of the media tested, even though differences in the frequency of caulogenesis were observed. When "Hansen 536" leaves were cultured on media C and D (Table S1), a low percentage of explants (about 15%) produced brownish calli (Figure 6 b, c) after 10 – 12 weeks of culture. Explants placed on media A, B, and E (Table S1) produced a high percentage (about 90%) of cream-colored calli after 10–12 weeks of culture (Figure 6 d – f). The cream-colored calli were transferred to a PGR-free medium (Procedure S4) in anticipation of the development of proembryonic masses and eventually somatic embryos but the calli turned brown and became necrotic 4 weeks after transferring to the PGR - free medium (Figure 6 g).

SE from petals and anthers

One-year-old dormant cuttings of the peach rootstock "GF677" (*P. persica x P. amygdalus*) were used for induction of SE from unopened flower petals and anthers (Figure 6 h, i). When petals and anthers with filaments were cultured on PAM (Procedure S5), both explants produced a significant percentage (about 71% and 96%, respectively) of cream-colored calli after 10 – 12 weeks of culture (Figure 6 j, k). The cream-colored calli were transferred onto PGR - free medium and turned brown and became necrotic after 4 - 6 additional weeks. When anthers with attached filaments were cultured on PIV medium (Franks et al., 1998), cream-colored calli formation was about 97% after approximately 3 months of culture (Figure 6 l). However, as observed in the previous trials, they turned brown and became necrotic after 6 weeks of culture in PGR - free medium. There was no calli formation from anthers and filaments on MSI medium (Dhekney et al., 2016) and the explants shriveled and dried up (Figure 6 m).

Our results showed that none of the media tested induced the embryogenic potential in the somatic cells treated. Studies on the evaluation of endogenous hormonal levels of peach adult tissues such as leaves and flowers would be helpful in assessing the appropriate synthetic hormonal stimulus capable of inducing SE in peach *in vitro* cultures. Concerning the choice of the source of explant used to obtain peach somatic embryos, various aspects must be considered. In general, the age of tissue has an impact on SE in horticultural plants (Ji et al., 2011). The endogenous hormonal balance of *P. persica* cotyledons influenced their capacity to pass through from the differentiated to the embryogenic stage (Perez-Jimenez et al., 2013). Ji et al. (2011) remarked that a young tissue at early stages of development, with a high level of basal metabolism, seems to be more susceptible for SE induction compared to an older differentiated tissue. In fact, SE induction can occur only if a differentiated cell regains its totipotency (Pasternak et al., 2002). As reviewed by Druart (1999), SE induction in immature tissues occurs over a very short period during bloom and seed or embryo development. Thus, it would be worth trying to work on the stimulation of SE in adult tissues cultured *in vitro* (such as leaves, for instance), which apparently should not need to be treated in a strictly fixed period. Culture of petals and anthers at different developmental stages should be evaluated for SE production in peach, as has been carried out in species such as *Vitis* (Dhekney et al., 2016).

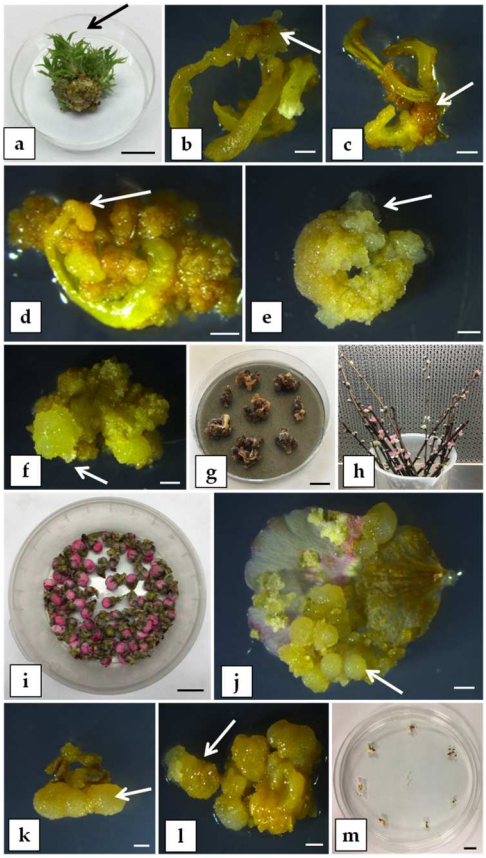

Figure 6. Somatic embryogenesis (SE) trials on peach mature explants. (**a**) *In vitro* meristematic bulk (MB) of "Hansen 536"; the arrow indicates the type of young leaf collected and used as starting explant in SE induction experiment (bar = 1 cm). Brownish calli (arrow) developed from "Hansen 536" leaves cultured on medium C (**b**) and on medium D (**c**); the images were taken after 3 months from the beginning of the experiment (bar = 2 mm). Cream-colored calli (arrow) developed from "Hansen 536" leaves cultured on medium A (**d**), on medium B (**e**), and on medium E (**f**); the images were taken after 3 months from the beginning of the experiment (bar = 2 mm). Necrotic calli from "Hansen 536" leaves cultured on plant growth regulator (PGR)-free medium (**g**) after 4 months from the beginning of the experiment (bar = 1 cm). Cuttings of peach rootstock "GF677" (**h**) and sterile unopened flowers of "GF677" (**i**) used as starting explants in the SE induction experiment (bar = 1 cm). Cream-colored calli (arrow) developing from petal (**j**) and anther with filament (**k**) of "GF677", both cultured on PAM medium after approximately 3 months from the beginning of the experiment (bar = 2 mm). (**l**) Cream-colored calli formation (arrow) from "GF677" anther with filament cultured on PIV medium (Franks et al., 1998) (bar = 2 mm). (**m**) "GF677" anthers with attached filament cultured on MSI medium (Dhekney et al., 2016) for 3 months (bar = 1 cm).

3.2.2. Organogenesis from adult material

Leaf explants

We obtained high levels of *in vitro* adventitious root regeneration from leaves (Table 5). This contrasts with the diffculty of regenerating shoots from peach leaf explants. Adventitious rooting was produced from leaves excised from greenhouse or *in vitro*-grown peach plants on MS medium (Murashige and Skoog, 1962) with 9 – 12 µM NAA, with or without kinetin at 0.4 – 1.2 µM (Procedure S6). Higher numbers of roots were obtained when leaf explants were cultured in the dark. Kinetin levels of 3.6 and 10.8 µM inhibited rooting. Roots produced in the light were thick, long, and geotropic, while roots developed in the dark were thin and non-geotropic. Roots originated from vascular areas of the leaf pieces. Root meristems were evident within 14 days after culturing leaves from plants grown in the greenhouse.

Table 5. Adventitious rooting from leaf segments of greenhouse-grown bud-grafted plants and *in vitro*-grown peach seedlings

Genotype	Rooting (%)		Average root Number	
	Dark	Light	Dark	Light
Greenhouse-grown [a]				
"EVD 1"	40	5	6.5	2.7
"EVD 2"	35	0	3.7	0
"EVD 3"	47	0	2.3	0
"EVD 44288"	61	0	2.9	0
"Redglobe"	60	0	2.3	0
"Redhaven"	44	0	2.1	0
***In vitro*-grown [b]**				
"Coacalco OP"	92	35	7.0	2.6
"Rutgers Redleaf double haploid OP"	58	9	4.9	2.0
"Sihung Chui Mi OP"	73	19	5.1	4.1
"Nemaguard OP"	84	25	6.6	3.4
"Indian Cling OP"	90	6	5.3	1.0

[a] Within greenhouse-grown leaves, ANOVA indicated that light was a significant factor at p = 0.0001 for both percentage rooting and number of roots.

[b] Within *in vitro*-grown leaves, ANOVA indicated that light was a significant factor for percentage rooting at p = 0.0001 and for number of roots at p = 0.012.

Effcient shoot proliferation and axillary meristematic explants

The utilization of axillary shoot meristematic tissues as gene delivery targets may facilitate the development of a reproducible and reliable transformation system in peach. One of the major challenges of this approach is the slow rate of cell growth and shoot proliferation and the limited availability of proliferative or meristematic tissues for *Agrobacterium* infection.

To address this bottleneck, we developed and tested an improved shoot proliferation system using many peach varieties including open-pollinated Bailey (*P. persica* "Bailey-OP") (Figure 7 and Procedure S7). Using the established conditions, a typical single shoot tip can form a cluster with 50 to 100 individual shoots in 2 months (Figure 7 b, c). We further incorporated the use of volatile compounds (VCs) of *Cladosporium sphaerospermum* strain TC09 to improve the otherwise previously reported long - term and laborious root induction process involving peach *in vitro* shoots (Hammerschlag et al., 1987; Li et al., 2019). As demonstrated in Figure 7, *C. sphaerospermum* dramatically enhanced root growth in "Bailey-OP" *in vitro* shoots. On average, up to 87% of VC - treated rooted shoots acclimatized successfully to the soil conditions and developed into robust plants as compared to 38% acclimatization rates among control shoots without VC treatment. Thus far, over 30 peach genotypes/varieties have been tested and many yielded similar rates of shoot proliferation.

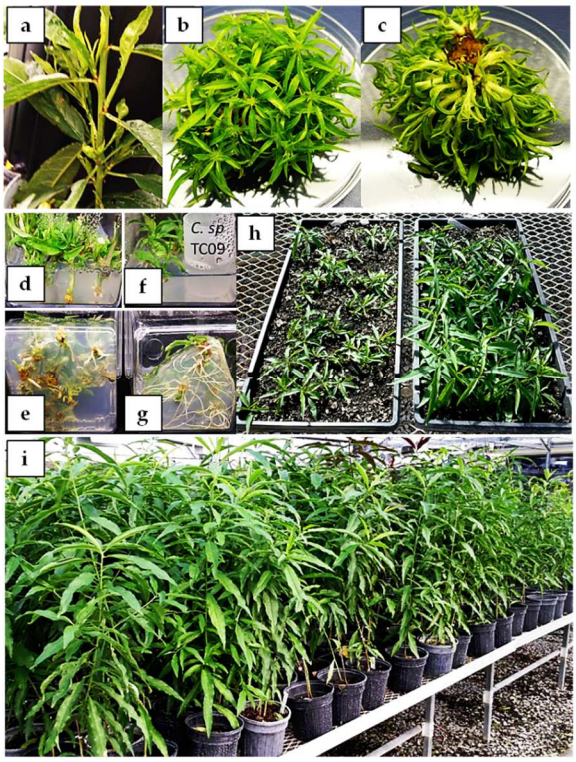

Figure 7. *In vitro* micropropagation of peach (*P. persica* L.) rootstock cv. "Bailey-OP". **(a)** Shoot culture explant source from greenhouse-grown plant. Top **(b)** and bottom **(c)** views of an individual *in vitro* shoot cluster of peach rootstock "Bailey-OP" derived from a single shoot explant after 50 days of cultivation on LP medium supplemented with 4.5 µM 6-benzylaminopurine (BAP) and 0.5 µM IBA. Rooted shoot without **(d, e)** or with **(f, g)** exposure to VCs emitted by *C. sphaerospermum* isolate TC09 for 10 days. **(h)** Growth of plantlets previously treated without (tray on left, control) and with (tray on right) volatile compounds (VCs) 1 month after transplanting to soil in 1020 trays. In this representative comparison, control tray contains 36 surviving plants out of 100 transplanted plants. The tray on right side has 46 surviving plants out of 52 transplanted plants. **(i)** Normal growth and development of *in vitro* propagated "Bailey-OP" plants 3 months after transplanting.

Agrobacterium-mediated genetic transformation experiments were conducted with the strains GV3101 and EHA105 harboring the pSGN binary plasmid (Li et al., 2008), containing the enhanced *gfp* (*egfp*) and *npt*II marker genes. Shoot explants were prepared by carefully cutting across the apex region of axillary buds in each shoot of 2–3 cm in length to expose the meristematic tissues for *Agrobacterium* infection. Following transformation and 1-week cultivation, GFP expression was not detected in control explants without *Agrobacterium* exposure (Figure 8 a, b). On the other hand, over 90% infected shoots showed transient GFP expression in one or more cut sites (Figure 8 c, d). Although further microscopic examination needs to be conducted to provide proof, vascular tissues seemed to be more prone to infection, as indicated by the ring form of GFP-expressing cells across the infection site (Figure 8 d). Noticeably, the lack of GFP expression in the meristematic dome region still needs further investigation. One month after culturing on kanamycin-containing (100 mg/L) shoot development medium (Procedure S7), no callus growth was found in any control explants (Figure 8 e, f), while GFP-stable expressing calli developed from *Agrobacterium*-infected explants (Figure 8 g, h). Putative transgenic shoots were also recovered. However, GFP expression was not detected in leaves of shoots that survived 3 to 4 months of kanamycin selection (100 mg/L). This indicated that the recovered shoots were non-transgenic escapes. The main reason could be the significantly reduced selection pressure on target cells due to the filtering effect of the relatively large-size parental stem section. The growing shoots may also have developed from un-exposed, pre-existing shoot primordia around the cut site. On the other hand, we tested excised individual axillary buds, with or without slicing through the middle region, using similar transformation conditions, and

only recovered non-organogenic transgenic calli at low frequencies. Quite often, the majority of these excised small explants quickly turned necrotic and succumbed to *Agrobacterium* overgrowth.

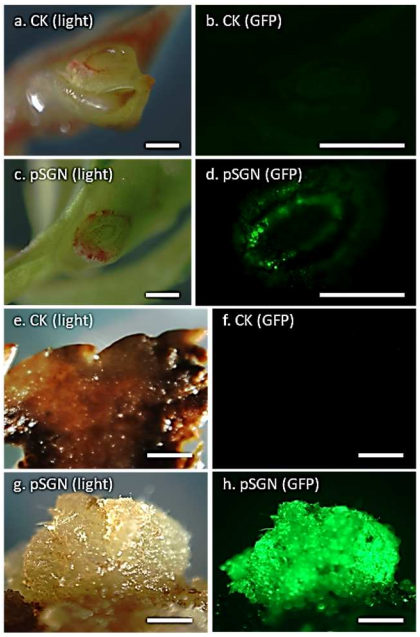

Figure 8. Transient and stable GFP expression detected in peach (*P. persica* cv. "Bailey-OP") shoot explants transformed with the binary vector pSGN. Detection of transient GFP expression in non-transformed (white light (**a**) and UV light (**b**)) and transformed (white light (**c**) and UV light (**d**)) shoot explants 1 week after transformation. Detection of stable GFP expression in callus tissue derived from control (white light (**e**) and UV light (**f**)) and transformed (white light (**g**) and UV light (**h**)) shoot explants after 1 month in selection with 100 mg/L kanamycin (bar 2 mm).

Nodal explants

Following Procedure S8, *in vitro* "Bailey-OP" shoots were used as the source of nodal explants. For the transformation experiments, the disarmed *A. tumefaciens* EHA101 strain harboring the binary plasmid pVNFbin was used.

The addition of 20 mg/L kanamycin or 20 mg/L paromomycin to the regeneration medium significantly ($p < 0.01$) reduced regeneration compared to the controls without the addition of antibiotics (Figure 9 a). Statistical differences between regeneration of non-infected and infected explants within the same treatment were not found for any of the different selection strengths applied (Figure 9 a). After 6 weeks from the beginning of the experiment, all green, healthy buds were isolated and placed onto a meristem development medium (Perez-Tornero et al., 1999) supplemented with 15 mg/L kanamycin. They were subcultured onto fresh medium every 2 weeks. All buds regenerated from non-infected explants became chlorotic and died in 2–4 weeks. Some of the buds regenerated from the infected explants were able to survive longer during the selection process. Surviving buds were subcultured, and transformation evaluation was conducted by GUS assays and/or molecular tests (PCRs or Southern blots). On the basis of GUS assays, two chimeras were detected, as the blue staining was only located in a particular area of the bud (Figure 9 b). Molecular tests revealed that none of the surviving shoots by the end of the selection procedure were transgenic. The two chimerical shoots originated from the experiment where 20 mg/L paromomycin was applied for selection, reaching 1.7% transformation effciency. On the basis of these results, it seems that the selection applied was too low, since non-transgenic escapes and chimeras survived. Further studies should be conducted with more stringent selective conditions or applying a gradual increasing selection to be able to dissociate chimeras and recover completely transformed shoots.

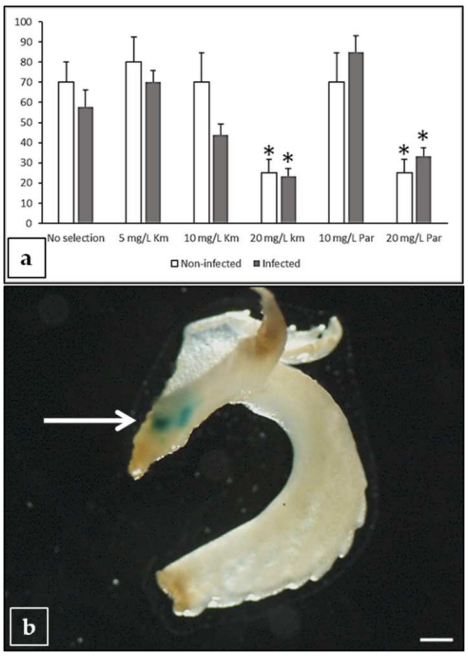

Figure 9. Regeneration and transformation from peach nodal explants. (**a**) Effect of antibiotics on adventitious regeneration from non-infected explants and EHA101 (pVNFbin)-infected explants. Regeneration data were collected at 6 weeks from the beginning of the experiment. Asterisks indicate statistical significance ($p < 0.01$) compared to the "no selection" treatment according to Pearson's chi-test. A total of 565 explant "Bailey-OP" were used for this study. Experiment was repeated at least twice. (**b**) Chimerical regenerated shoot showing GUS activity (arrow) (bar = 1 mm).

Meristematic bulks

An adventitious shoot regeneration method, based on the generation of a meristematic bulk (MB) from shoot tips, has been applied successfully in different peach cultivars ("Big top", "Zaitabo", "UFO-3", "Maruja", "Flariba", and "Alice Bigi") and in *P. persica x P. amygdalus* rootstocks ("GF677", "Garnem", and "Hansen 536") (Girolomini et al., 2012; Perez-Jimenez et al., 2012; Sabbadini et al., 2019, 2015). Furthermore, this method allowed the regeneration of transgenic plants of the peach-almond hybrid "GF677" (Sabbadini et al., 2015). A similar protocol has been applied to other perennial plant species, such as grapevine and blueberry (Cappelletti et al., 2016; Mezzetti et al., 2002), showing its versatility and potential for *in vitro* regeneration and/or genetic transformation of fruit species.

To improve the previously described procedure in peach (Sabbadini et al., 2015), two factors that may affect *Agrobacterium*-mediated transformation were further studied: (i) the addition of phenolic compounds such as acetosyringone (AS), and (ii) the utilization of ethylene inhibitors such as STS. The addition of AS increased *Agrobacterium*-mediated transformation in apricot (Laimer da Camara Machado et al., 1992; Petri et al., 2004) and almond (Costa et al., 2006; Miguel and Olivera, 1999). Furthermore, endogenous plant level of ethylene reduces *Agrobacterium*'s ability to transfer the T-DNA into plant cells (Nonaka et al., 2008).

Transformation experiments were performed in the peach x almond hybrid rootstock "Garnem" and the peach cultivars "UFO-3" and "Alice Bigi". Two disarmed *A. tumefaciens* strains C58 (pMP90) (Koncz and Schell, 1986) and EHA105 (Hood et al., 1993), both carrying the binary plasmid pBin19-*sgfp* (Chiu et al., 1996), were utilized. AS was added to the bacterium culture medium and transgenic cells were selected with 50 mg/L kanamycin. Transformation was monitored through GFP expression. Two different explants were used for transformation: (1) the basal part of the shoots, which would produce the MB, and (2) slices of the MB. In both cases, GFP was only expressed transiently, and stable transformation was not detected. A pre-culture in darkness after infection enhanced the number of cells showing GFP signal and the stability of them (data not shown). In this study, the effect of the addition of AS to the bacterium

medium was genotype dependent; it produced no effect in "Garnem", was counterproductive in the case of "Alice Bigi", and generated different results depending on the *Agrobacterium* strain in "UFO-3" (not shown).

Additional trials were performed to improve the previous transformation results obtained in "Hansen 536" MBs (Sabbadini et al., 2019). The two factors studied were (i) the addition of AS in the co-culture medium at concentrations of 0, 50, 100, and 200 µM, and (ii) STS added during the co-culture period and/or in the regeneration/selection medium for the first 2 weeks after *Agrobacterium* infection. Transformation trials were carried out following the protocol described by Sabbadini et al. (2019) with MB slices used as starting explants (Figure 10 a, b) and the EHA105 *A. tumefaciens* strain harboring a construct with the *npt*II and *egfp* genes. The addition of AS, at the concentrations tested, or the different STS treatments assayed did not influence the transformation effciency at 3 months post-infection compared to the controls. From these experiments, transformed shoots were not obtained and only portions of stably transformed callus expressing *egfp* were observed (Figure 10 c, d), similar to previously obtained results (Sabbadini et al., 2019).

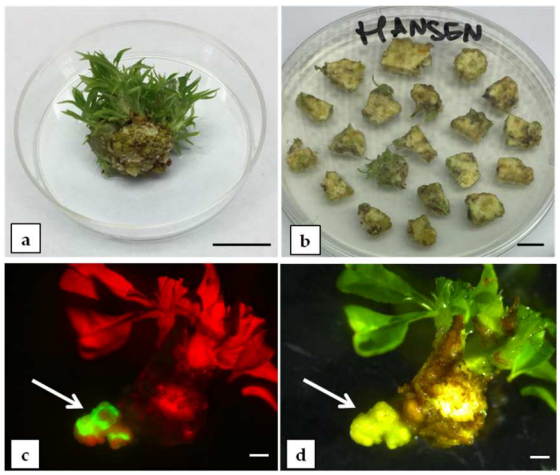

Figure 10. Organogenesis trials on peach meristematic bulks (MBs). (**a**) MB of "Hansen 536" (bar = 1 cm). (**b**) Slices (1 cm2, 2 mm thick) obtained from "Hansen 536" MBs used as starting explants for *A. tumefaciens*-mediated transformation trials (bar = 1 cm). "Hansen 536" stably transformed callus-expressing eGFP (arrow) observed under UV light (**c**) or under white light (**d**)**.** Photographs taken at 3 months post-infection (bar = 2 mm).

4. Possible solutions to the bottleneck

In order to produce transgenic plants successfully, there should be an overlap between peach tissue cells able to regenerate adventitious shoots and those amenable for transformation. In general, the lack of an effcient adventitious regeneration protocol is the limiting factor for gene transfer technologies in fruit tree species. The results presented in this manuscript, together with previously published studies, show that adventitious shoot regeneration, while genotype-dependent, does not seem to be the major problem for this species. Both the integration of the transgene/s into the plant genome and then the recovery of uniformly transformed plants is problematic. Even with regeneration rates as high as 40–100%, the production of the transgenic shoots has either not been possible or has been successful only with an extremely low effciency, therein frequently producing shoots that are chimeric for transformation. Considering the extremely low rate of transformation (zero most of the time) and the regeneration of chimeric shoots as the main bottleneck to genetic engineering of peach, this section discusses different possibilities to improve *Agrobacterium*-mediated transformation and selection of transformed tissues of peach.

In plant transformation, an appropriate selection protocol is essential to obtain transgenic plants. Most of the peach transformation protocols published include aminoglycoside antibiotics for selection. It is known that this class of antibiotics can interact with both the membrane of cells and their receptors and with components of the culture medium

(Ca^{2+}). Their activity is also affected by light, pH, and/or temperature (Padilla and Burgos, 2010). Antibiotic concentration decreases in the medium due to degradation in the vicinity of transgenic cells capable of inactivating them (Rosellini et al., 2007), and thus it has been frequently suggested as a subculture to avoid escapes and chimeras. Researchers should take into account all these particularities of antibiotics to apply the most appropriate concentrations to plant tissues at each step of development. In peach, it would be important to determine which concentrations are limiting the growth of the initial explant, as well as the initiation and development of meristems and shoots, in order to establish a selection protocol coupled to organogenesis and organ development. The use of alternative selective marker genes could also be part of the solution. In peach, we have tried the *bar* and *ipt* genes in mature seed hypocotyl sections without success. However, these selective marker genes could be appropriate for other peach explants. Moreover, selection strategies based on mannose or hygromycin have shown to be amenable for other *Prunus* spp. (Sidorova et al., 2017; Tian et al., 2009; Wang et al., 2013). Further studies that are focused on selection methods should be carried out, including combining visual marker genes, such as GFP, and histological studies to verify the different competences of cells in transformation and regeneration processes.

New studies on genetic factors determining peach cell transformation recalcitrance could help to find molecular solutions to develop effcient protocols. During the past 45 years, model plant systems, such as *Arabidopsis*, have been exploited to describe and understand the T-DNA transfer and insertion into the host plant genome at a molecular level, due to their ease of expressing the transgene, both in transient and stable transformation. From our perspective, studies reporting the reduction of transient and/or stable transformation effciency in some *Arabidopsis* ecotypes are of particular interest, including examples of recalcitrance to *Agrobacterium* transformation (reviewed by Hwang et al., 2017). Collectively, these reports showed how the *Agrobacterium*-mediated gene delivery system in plants could fail at any step of the process, including the first physical contact of bacterium to plant tissue, delivery of T-DNA from the bacterial cytoplasm up to its importation, or integration and expression in the plant nucleus. It is interesting to note that peach trees are quite susceptible to crown gall (Zoina and Simeone, 1989). Hwang et al. (2017) reviewed the role of several plant key genes participating in all these events, indicating the possibility that their focused over-expression and/or downregulation enhanced transformation rates of both recalcitrant and susceptible model plant lines. We suggest that this approach may be promising and suitable to almost all the species of the genus *Prunus*, which are (with the exception of plum) recalcitrant to *Agrobacterium*. Genes affecting plant regeneration itself could be useful in the road to improve peach genetic transformation; an example of this is the ectopic expression of the corn meristem identity gene KNOX1 in plum plants, which significantly improved adventitious shoot regeneration from plum leaf explants (Srinivasan et al., 2011).

The *Agrobacterium*–host interaction is a war of cell survival, in which the host defense system combats the intruding pathogen. As suggested by a pioneering work carried out by Dunoyer et al. (2006), plant defense reactions rely on the induction of RNA silencing pathways to limit the expression of bacterial T-DNA. Therefore, to enhance peach competence for *Agrobacterium*-mediated transformation, alternative strategies may consist in attenuating the reaction of plant defense responses in infected tissues. As master gene silencing regulators, microRNAs are involved in many developmental processes, such as organogenesis, somatic embryogenesis, and resistance against pathogens (Martin et al., 2010; Willmann and Poethig, 2007). Some years ago, researchers were committed to build microRNAs libraries with the main aim of evaluating how these molecules alter their expression profiles during *in vitro* developmental stages. These results are crucial for the optimization of more suitable *in vitro* culture conditions, especially for recalcitrant species. In particular, several studies reporting microRNA expression patterns, both from model plant and some cultivated crop tissues, during different bacterial infections showed that bacterial elements trigger

the up- or downregulation of specific microRNAs, which suppress or induce key negative or positive regulators of the host defense (reviewed by Kamthan et al., 2015). In addition, key microRNAs involved in somatic embryogenesis (Siddiqui et al., 2019) suggest that increasing our understanding about the role of these molecules could also contribute to improved gene transfer protocols based on SE. In peach, several microRNAs involved in response to different stress conditions have been identified (Barakat et al., 2012; Eldem et al., 2012; Gao et al., 2012; Li et al., 2017; Luo et al., 2013; Zhang et al., 2016); nevertheless, microRNA expression profiles from *Agrobacterium*-mediated transformed or infected tissues have not been built to date. Gaining an understanding of the role of microRNAs and their target mRNAs in preventing genome modification may be useful in elucidating appropriate *in vitro* stimuli capable of inducing effcient *Agrobacterium*-mediated transformation in recalcitrant *in vitro* cultures, including peach. Moreover, the addition of antioxidants to cope with toxicity of reactive oxygen species (ROS) generated as a result of the *Agrobacterium* infection may improve peach transformation as has been described in other plant species such as Mexican lime and tomato (Dan et al., 2014; Dutt et al., 2011).

Lastly, following its first detection in the middle of the 20th century (Riker et al., 1930; White, 1972), *A. rhizogenes*-mediated adventitious hairy root disease in dicotyledonous plants has been widely investigated and used as a transgenic tissue generation system in plant biotechnology, mainly as an alternative option for *A. tumefaciens* gene delivery in plants (Hwang et al., 2017). As reviewed by Giri and Narasu (2000), adventitious shoot regeneration can occur directly from transgenic roots or by moving them to regeneration medium. As recently shown, the transgenic hairy root phenotype has been induced in different peach explants such as leaves, hypocotyls, and shoots using *A. rhizogenes* strain MSU440 (Xu et al., 2020). The main goal of this study was to optimize a reproducible *A. rhizogenes*-mediated transformation protocol for gene function and genetic engineering studies in peach. Although adventitious regeneration from *in vitro* root cultures is diffcult, an effcient shoot regeneration method from roots in *P. persica* could be a further approach for peach genetic improvement, as the production of peach transgenic plants through *A. tumefaciens* has been arduous to date.

The utilization of novel DNA delivery methods in peach should be further studied. In recent years, nanoparticles have been extensively utilized in many areas of research (reviewed by Wang et al., 2016). Successful nanoparticle-mediated introduction of DNA plasmids into plant cells at relatively high effciencies has been demonstrated (e.g., Demirer et al., 2019; Doyle et al., 2019). The methodology is relatively simple and may offer certain advantages such as the absence of phytotoxicity and high target cell coverage.

5. Conclusions

Many regeneration protocols are available from different type of peach tissues, some of them demonstrating a high effciency. Nevertheless, regeneration has not led to the reliable production of uniformly transgenic peach plants. In general, with regeneration approaches that involve adventitious organogenesis, the main issue remains the selection procedure for obtaining non-chimeric regenerated shoots, while the limit of SE is the development of effcient regeneration protocols, in particular from adult tissues. Peach immature cotyledons allowed effcient shoot regeneration through organogenesis and SE, but with low transformation rates.

Protocols for the development of transgenic peach cultivars are needed to apply new biotechnological tools that can help to resolve important problems affecting peach cultivation, in order to increase sustainability, resilience, and quality. Future and current fruit tree breeding programs should integrate classical- and biotechnologies. For the development of a reliable peach transformation system, the key issues to be researched are the low effciency of *A. tumefaciens*-mediated transformation, the low level of correspondence between cells competent for transformation and

those that have regeneration competence, and the high rate of chimerism in the few shoots that are produced following transformation procedures.

While we currently have focused on the scientific aspects of developing improved peach cultivars through genetic engineering, a major impediment to the application of this and other novel genetic technologies in applied fruit breeding is, in general, the lack of clear, effcient, and science-based regulatory regimes.

6. Supplementary material

Procedure SM1: Adventitious bud regeneration from peach immature cotyledons

Immature fruits of 45-50 days after blooming are collected and surface disinfected with 70% (v/v) ethanol. Immature cotyledons are taken from the seeds in a laminar hood and placed on Preculture Medium with the adaxial face down for 7 days in the dark at 24 ± 1 °C. Preculture Medium consists of QL salts and vitamins (Quoirin and Lepoivre, 1977), 3% sucrose, 5 µM 2, 4-D, 0.5 µM BAP and 0.65% bactoagar. Following the preculture, explants are placed on Regeneration Medium [QL salts and vitamins, 3% sucrose, 12.5 µM thidiazuron (TDZ), 0.4 µM NAA, 60 µM silver thiosulphate (STS), 0.7% bactoagar] and transferred to a 16/8 h light/dark cycle, light intensity of 45–50 µE $m^{-2}s^{-1}$, and a temperature of 24 ± 1 °C. Adventitious buds start appearing as direct regeneration after 4-5 weeks, normally as cluster throughout the whole adaxial surface of the explant (Figure 1 a, b, c).

As buds/clusters appear, follow a two-step procedure previously described (Perez-Tornero et al., 1999) to rescue and elongate them. Briefly, clusters/buds are firstly cultured on a Meristem Development Medium consisting of QL salts and vitamins (Quoirin and Lepoivre, 1977), 2% sorbitol, 7.09 µM BAP, 0.05 µM indole-3-butyric acid (IBA) and 0.7% bactoagar. After 2-4 weeks, buds/clusters have grown up as a rosette of 0.5-1.0 cm wide. At this point, rosettes are transferred to Shoot Elongation Medium consisting of QL salts and vitamins, 2% sorbitol, 3.5 µM BAP, 5.8 µM gibberellic acid (GA_3), 0.05 µM IBA and 0.7% bactoagar. As shoots elongate from the rosette and reached 2-3 cm long, they are excised and placed in Rooting Medium previously designed for apricot. Rooting Medium consists of QL macros (Quoirin and Lepoivre, 1977), DKW micros and vitamins (Driver and Kuniyuki, 1984), 2% sucrose, 1.5 mM $CaCl_2$, 0.8 mM phloroglucinol, 2.95 µM IBA, 29.6 µM adenine and 0.7 % bactoagar. Adventitious roots start being visible after two weeks of culture in Rooting Medium and with two more additional weeks, shoots show a well-developed root system (Figure 1 d). All media pH are adjusted to 5.7 and sterilized in an autoclave at 121°C for 20 min. Acclimatization of shoots is accomplished following standard procedures (Figure 1 e) (Petri et al., 2012).

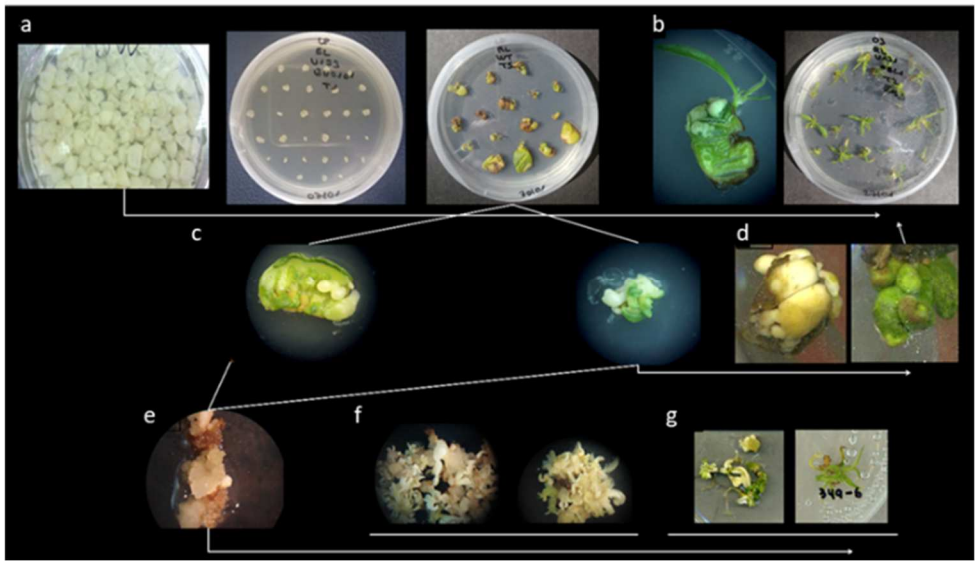

Figure SM1. Somatic embryogenesis (SE) in peach from immature cotyledons. In a regular procedure designed for direct organogenesis, immature cotyledons obtained 50-70 d post bloom **(a)** are led to direct budding in LP modified medium (Gentile et al., 2002) **(b)** with 6-benzylaminopurine (BAP) (5.0 µM) and α-naphthaleneacetic acid (NAA) (between 3.0 and 5.0 µM) in a process that requires between 3 and 6 months. This treatment will lead to the formation of white-egg shape structures **(c)** that can be induced to organogenesis by additional culturing for 30-60 d in LP medium (Gentile et al., 2002) supplemented with 1.0 µM 2, 4-Dichlorophenoxyacetic acid (2, 4-D) **(d)**. In the process, callus formation **(e)** will be also obtained whose lead to continuous somatic embryo formation **(f)** by culturing these explants in LP medium supplemented with sucrose (4%). This latter procedure **(e + f)** can be kept for at least one year. Although genetic transformation was not evaluated, this procedure allowed whole plant production **(g)**. All the procedures are carried out under a 16 h light photoperiod at 22 ± 1 °C.

Procedure SM2: Adventitious bud regeneration from peach mature hypocotyl sections

Mature seeds stored at 4 °C are used. Seeds can be stored for several years. For seeds disinfection, the endocarp is removed with a nutcracker, and seeds are immersed in a beaker containing a solution of 20% commercial bleach (commercial bleach = 6.15% sodium hypochlorite) and 0.02% Tween-20 for 20 min and rinsed three times with sterile water in a laminar flow bench. Disinfected seeds are soaked in sterile water overnight at 4 °C. The seed coat is removed with the aid of a scalpel, the radicle and the epicotyl are discarded, and the hypocotyl is sliced into 2–4 cross sections (0.5–1 mm), which are placed on the regeneration medium. Regeneration medium consists of QL basal salts (Quoirin and Lepoivre, 1977), MS vitamins (Murashige and Skoog, 1962), 2% (v/v) sucrose, 7.5 µM TDZ, 0.25 µM IBA, 0.5 µM aminoethoxyvinylglycine (AVG) and 0.7% (v/v) purified agar (Sigma). The pH is adjusted to 5.8 before autoclaving at 121°C for 20 minutes. Explants are cultured in dark conditions for 2 weeks before transferring them to a 16/8 h light/dark photoperiod, light intensity of 45–50 µE m^{-2} s^{-1}, and a temperature of 24 ± 1ºC. First buds start appearing after 4 weeks from the beginning of the experiment as direct adventitious regeneration, and additional buds appeared in the 2-3 following weeks. As buds/clusters appear, follow the two-step procedure described in Procedure SM1.

Procedure SM3: Adventitious bud regeneration from seed-derived internodes

Seeds are induced to germinate without cold stratification by soaking overnight in a 1:1 solution of 500 ppm BAP and 500 ppm GA_3 and, subsequently, germinated *in vitro* in the dark on C2D medium (Chee and Pool, 1982) with the addition 40.0 µM BAP, 5.0 µM TDZ, 5.0 µM IBA, MS vitamins (Murashige and Skoog, 1962), 100 mg/L myoinositol, 3% sucrose and 0.6% Bacto agar. Upon germination (12 to 17 days), the internode from the basal section of the germinating epicotyl immediately above the point of attachment with the cotyledon is sliced into 2-3 mm sections

and used as primary explant. The Regeneration Medium consists of LP macro and micronutrients (LP1) (Quoirin and Lepoivre, 1977), MS vitamins (Murashige and Skoog, 1962), 2.5% sucrose and 100 mg/L myoinositol and 0.6% Bacto agar. Medium is supplemented with 1.0 µM NAA and 10.0 µM BAP. All media pH was adjusted to 5.8 and sterilized in an autoclave at 121°C for 20 min.

Procedure SM4: SE from in vitro leaf explants

Leaves are cultured with the abaxial side in contact with the SE induction media. The induction media consisted on MS basal salts and vitamins (Murashige and Skoog, 1962), 20 g/L sucrose, and 7 g/L plant agar (Duchefa Biochemie, Italy), pH 5.7-5.8, supplemented with several combinations of plant growth regulators (PGRs) and amino acids (Table SM1). Leaves were incubated in darkness at 24 ± 1°C for seven weeks and then exposed to light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for five weeks.

Explants placed on media A, B and E (Table SM1) produce cream-colored calli after 10-12 weeks of culture. These calli are then transferred to a PGRs-free medium consisting of MS basal salts and vitamins (Murashige and Skoog, 1962), 20 g/L sucrose, 7 g/L plant agar (Duchefa Biochemie, Italy), and 0.5 g/L activated charcoal (Duchefa Biochemie, Italy), pH 5.7-5.8. Cultures are maintained under the light and temperature regime described above.

Table SM1. Different concentrations and combinations of PGRs and amino acids used in leaves SE induction media

	PGRs and amino acids (µM)					
SE induction media	**BAP**	**NOA**	**NAA**	**2, 4-D**	**GA_3**	**Proline (mM)**
A	2.2	0.5				
B	8.9		10.7			
C	2.2	0.5			0.4	2.6
D	4.4		0.3		0.4	2.6
E				14		2.6

Procedure SM5: SE from peach petals and anthers

Petals and anthers, from sterile unopened flowers, with filaments intact are carefully removed and cultured with the adaxial side (for the petals) in contact with the SE induction medium, whereas anthers are horizontally placed on the medium. The induction medium for petals and anthers (PAM) consists of MS basal salts and vitamins (Murashige and Skoog, 1962), 20 g/L sucrose, and 7 g/L plant agar (Duchefa Biochemie, Italy), pH 5.7-5.8, supplemented with 14.0 µM 2, 4-D and 0.22 µM BAP. Both explants are then maintained in darkness at 24 ± 1°C for 5-7 weeks before transferred them to light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C during 5 additional weeks.

Additionally, two media, named as PIV (Franks et al., 1998) and MSI (Dhekney et al., 2016), used for embryogenic culture induction from *Vitis* spp. anthers, were tested for 'GF677' anthers. PIV medium (Franks et al., 1998) consisted of Nitsch and Nitsch basal salts (Nitsch and Nitsch, 1969) and B5 vitamins (Gamborg et al., 1968), 60 g/L sucrose, and 3 g/L Phytagel (Sigma-Aldrich), pH 5.7 supplemented with 8.9 µM BAP and 4.5 µM 2,4-D. MSI medium (Dhekney et al., 2009) containing MS basal salts and vitamins (Murashige and Skoog, 1962), 0.1 g/L myo-inositol, 20 g/L sucrose, and 7 g/L plant agar (Duchefa Biochemie, Italy), pH 5.8 supplemented with 4.5 µM BAP and 5 µM 2, 4-D. Both cultures were then incubated in darkness at 24 ± 1°C for seven weeks and then exposed to light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for five weeks. When cream-colored calli appear, they are transferred to PGRs-free medium. Cultures are maintained under light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ±1°C for six weeks.

Procedure SM6: In vitro organogenesis of roots from peach leaf explants

Leaves from greenhouse-grown seedlings and mature, greenhouse-grown plants are disinfested by immersion for 30 seconds in 70% ethanol and then soaked for 10 min in a solution of 0.8% sodium hypochlorite with 2 drops per liter of Tween 20. They are then rinsed 3 times in sterile distilled water. Leaves are cut into transverse strips with a scalpel and explanted abaxial surface down onto treatment media. Each leaf produced 5-6 segments with the segment(s) from mid-leaf ranging in size from approximately 6 x 8 mm to 9 x 15 mm. Several punctures are made through each leaf piece. The growth regulator treatments added to MS medium (Murashige and Skoog, 1962) included combinations of kinetin at 0, 0.4, 1.2, 3.6, and 10.8 µM; with IBA or NAA at 0, 3.0, 6.0, 9.0 and 12.0 µM. Growth regulators were added to the basal medium prior to autoclaving. Root development was evaluated after 5 weeks of culture in the dark or light (45-50 µE $m^{-2}s^{-1}$ with a 16h photoperiod) at 24 °C.

Procedure SM7: Improved peach in vitro shoot proliferation

Greenhouse-grown plants/cuttings are preferred as explant source for culture initiation, due to less incidences of microbial contamination and immediate robust regrowth, once shoot tips were placed on shoot proliferation medium. Shoots are cultured in LP basal medium (Quoirin and Lepoivre, 1977) lacking myo-inositol and supplemented with vitamins 1.0 mg/L thiamine-HCL, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine-HCL, 4.0 mg/L glycine, 0.2 mg/L biotin and 2.0 mg/L Ca-pantothenate, phytohormones 4.5 µM BAP and 0.5 µM IBA, 3% sucrose, 0.6% Sigma agar (Sigma-Aldrich, St. Louis, Missouri, USA, A7921) and pH value adjusted to 5.8. Medium is sterilized in an autoclave at 121°C for 20 min. Cultures are maintained at 25°C under a 16 h photoperiod (50 µE $m^{-2}s^{-1}$) and subcultured to fresh medium every three weeks.

A two-step procedure is employed to improve *in vitro* shoots rooting and establishment in the greenhouse. *In vitro* shoots, of more than 2 cm length, are first transferred to LP medium (lacking myo-inositol) supplemented with 5.0 µM IBA and cultured for 10 days to induce root primordia. After that, to encourage root and shoot development, 5-7 shoots are moved to each Magenta GA7 vessels containing PGRs-free LP medium with a culture tube closure containing the fungus *Cladosporium sphaerospermum* strain TC09 (Figure 7 f, g). Cultures are maintained at 25°C under a 16 h photoperiod (50 µE $m^{-2}s^{-1}$) for 10 days. Afterwards, robust plantlets with well-developed root system are transplanted to 1020 plastic trays (11" W x 21.37" L x 2.44" D) containing potting soil mix (Metro Mix, 852-F52, Sun Gro® Horticulture, Seba Beach, Canada) (Figure 7 h). Trays are covered with translucent plastic covers and kept under a shaded area for two weeks. Acclimatized plants are then transplanted to 6-inch pots containing similar soil mix for further growth and development in the greenhouse (Figure 7 i).

Details of fungal culture initiation and preparation are described previously (Li et al., 2019). Briefly, aqueous conidial suspension is prepared by first culturing the fungal conidia on MS plate for 1-2 weeks followed by collecting conidia in sterile 0.01% Triton X-100/water solution and adjusting density to 105 conidia per ml prior to use as inoculum. Aliquots of 5 ml warm, growth regulator-free MS medium are poured into open-end culture tube closures (Sigma C5791). Once solidified, 10 µl of TC09 conidial suspension is added onto the surface of the medium. One inoculated closure is placed in each Magenta GA7 vessel.

Procedure SM8: Bud regeneration from in vitro peach nodal explants

Three to four weeks-old *in vitro* shoots are used. Elongated, healthy, thick and green (non-lignified) shoots are chosen, and all buds are removed with a scalpel. Axillary buds are removed by running the scalpel parallel to the stem. Shoots are cut into segments containing, at least one node and placed onto regeneration medium, consisting of QL

macro and micronutrients, vitamins and organic compounds (Quoirin and Lepoivre, 1977), 3% sucrose, 0.7% Purified agar, 6.7 µM BAP and 0.05 µM IBA. The pH is adjusted to 5.7 before autoclaving at 121°C for 20 min.

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CHAPTER 3

RESEARCH SECTION

FACTORS AFFECTING THE REGENERATION, VIA ORGANOGENESIS, AND THE SELECTION OF TRANSGENIC CALLI IN THE PEACH ROOTSTOCK HANSEN 536 (*Prunus persica x Prunus amygdalus*) TO EXPRESS AN RNAi CONSTRUCT AGAINST PPV VIRUS USING MERISTEMATIC BULK AS STARTING MATERIAL[1]

1. Introduction

Traditional breeding techniques applied in the genetic improvement of *Prunus* species, especially peach and nectarines, are characterized by a significant level of heterozygosity. Additionally, laborious and time-consuming backcrossing cycles are a challenge, especially since agronomical traits are often recessive and/or controlled by several different genes (Carrasco et al., 2013; Petri et al., 2011). Virus resistance induction is among the primary goals in plant genetic improvement, and new breeding techniques (NBTs) represent a promising integrative tool to traditional methods, especially when applied on woody fruit species such as *Prunus* spp. (García-Almodóvar et al., 2014; Hily et al., 2007; Lemgo et al., 2013; Limera et al., 2017; Padilla et al., 2006; Scorza et al., 2013; Sidorova et al., 2017; Song et al., 2013). Since its first discovery, RNA Interference (RNAi) has become one of the most promising new strategies applied to induce resistance in plants against different kinds of pathogens, including viruses, fungi, and insects (Febres et al., 2008; Pessina et al., 2016; Wang et al., 2017; Zotti et al., 2017). This mechanism is known to regulate endogenous gene expression and has been discovered as a natural conserved defence strategy used by plants against virus infections (Frizzi and Huang, 2010; Huang and Li, 2018; Li et al., 2016; Molesini et al., 2012). It relies upon the production of double-strand RNAs precursors, which targets complementary transcripts in a sequence-specific manner, leading to the mRNA degradation or translation inhibition. The silencing molecules at the basis of RNAi mechanism has demonstrated to be capable of moving through the plant cell plasmodesmata or plant vascular system, for both short and long distances, respectively (Melnyk et al., 2011; Molnar et al., 2011; Reagan et al., 2018). In the case of woody plants species, this systemic transfer of silencing signal represents an advantage also when considered from a biosafety perspective, because the introduction of RNAi-based gene constructs directly in a rootstock, is expected to result in the transport of the silencing molecules to the non-modified scion (trans-grafting) (Haroldsen et al., 2012; Lemgo et al., 2013; Limera et al., 2017; Song et al., 2013; Zhao and Song, 2014). However, the application of these techniques still often relies on the stable introduction of the gene of interest into the plant genome, which presents a challenge for woody plant species as they are characterized by a high level of recalcitrance to transformation. Indeed, for an efficient gene introgression into the host genome, different parameters need to be considered and optimized, such as: (i) choice of starting plant material; (ii) *in vitro* regeneration medium composition (i.e., salt composition of basal medium, plant growth regulators combination, the gelling agent and the optional addition of antioxidant compounds, ethylene inhibitors, etc.); (iii) the bacterial strain chosen as vector, and the antibiotics used to contain bacterial persistence in the medium post transformation (when *Agrobacterium*-mediated transformation is exploited), and (iv) selection method applied to isolate putatively transformed lines and curbing the regeneration of escapes (non-transgenic shoots) (García-Almodóvar et al., 2014; Rai and Shekhawat, 2014; Sgamma et al., 2015). Different successful *in vitro* regeneration protocols have been developed for *Prunus* species, particularly in peach, using different starting tissues; among these reports, the majority used immature seeds and cotyledons as starting explants (Bhansali et al., 1990; Hammerschlag et

al., 1985; Pérez-Clemente et al., 2004; Scorza et al., 1990; Smigocki and Hammerschlag, 1991). However, regeneration from adult tissues is more recommended for clonally propagated crops to retain their desirable characteristics, especially for those with high heterozygosity as peach x almond hybrid, usually commercially used for peach plant propagation. *In vitro* shoot regeneration and/or callus induction from somatic tissues has been obtained in different peach genotypes starting from *in vitro* leaves (Declerck and Korban, 1996; Gentile et al., 2002; San et al., 2015; Zhou et al., 2010), stems, petioles and flower calyx (Pérez-Jiménez et al., 2013). Furthermore, a protocol developed by our group on table grape (Mezzetti et al., 2002) has been effectively utilized also for the *in vitro* regeneration via organogenesis, and genetic engineering of different peach varieties and rootstocks (Girolomini et al., 2012; Pérez-Jiménez et al., 2012). It is characterized by the induction of meristematic bulk (MB) formation starting from apices of *in vitro* cultured shoots; the MBs obtained are characterized by high competence for regenerating new shoots, providing the basis for a useful tool to genetically transform peach starting from adult tissues. A previous study in our group on the genetic transformation of the peach rootstock GF677 (*Prunus persica x Prunus amygdalus*) demonstrated the difficulty of transforming MBs belonging to this hybrid, indeed a transformation efficiency of 0.3% was achieved, which led to the production of two independent transgenic lines expressing an empty vector, named as hp-pBin19 (Sabbadini et al., 2015). A similar protocol has been applied by our group to the peach cultivar Big Top, showing once again its low efficiency in producing transgenic shoots (unpublished results).

In the present study, the aim was to optimize the regeneration protocol, via organogenesis, and *Agrobacterium*-mediated transformation of another commercial peach rootstock, Hansen 536 (*Prunus persica x Prunus amygdalus*) to introduce a hairpin gene construct against plum pox virus (PPV), which causes Sharka disease in several *Prunus* species (Scorza et al., 2013).

2. Materials and methods

2.1. Plant material and establishment of in vitro culture

In vitro proliferating cultures of the commercial peach rootstock Hansen 536 (*Prunus persica* x *Prunus amygdalus*) were established from shoot tips of 10-year-old Hansen 536 trees, grown in the greenhouse of Vitroplant Italia, Cesena, Italy. The sterilization protocol consisted of washing the plant materials in a solution of 1% (V/V) sodium hypochlorite for 15 min, rinsed three times with sterile water, and sub-cultured every 30 days on propagation medium. This medium is composed of MS basal salts and vitamins (Murashige and Skoog, 1962), 30 g L^{-1} sucrose, and 7 g L^{-1} plant agar (Duchefa Biochemie, Italy), with the addition of 4.4 µM BA and 0.05 µM NAA (pH 5.6–5.7).

Meristematic bulks (MBs) of Hansen 536 were induced and maintained following the protocols previously described (Mezzetti et al., 2002; Sabbadini et al., 2015). Briefly, MB induction was started from shoot tips, obtained from two-year-old *in vitro* proliferating cultures, which were cutted and cultured on induction medium (IM) composed of MS microelements and vitamins, 10.40 mM KNO_3, 5 mM NH_4NO_3, 1.47 mM KH_2PO_4, 1.62 mM $MgSO_4$ x 7H2O, 4.57 mM $CaNO_3$, 1.67 mM NaH_2PO_4, 30 g L^{-1} sucrose, 7 g L^{-1} plant agar (Duchefa Biochemie, Italy), pH 5.6–5.7. The explants were sub-cultured three times on IM medium enriched with 0.05 µM NAA and increasing concentration of BA (from 4.4 µM up to 13.2 µM), by transfering the apical dome at each sub-culture. MBs obtained were maintained on IM3 medium, which consisted of IM medium with the addition of 13.2 µM BA and 0.05 µM NAA and placed in the growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol/m2/s) provided by white fluorescent tubes.

2.2. Influence of different factors on the regeneration efficiency of Hansen 536 MBs

2.2.1. Effect of different basal media and plant growth regulator combinations

Thirty-day-old MBs of Hansen 536 cultured on IM3 were cut into slices (1 cm2, 2 mm thick) and utilized as starting explants for the regeneration experiments. MB slices were cultured in 9-cm diameter microboxes (Micropoli, IT) and used for testing 18 different media combinations, which differed in basal salts and vitamins composition: full-strength QL (Quoirin et Lepoivre, 1977), DKW (Driver and Kuniyuki, 1984) and modified WPM (Lloyd and McCown, 1980). Each of these media was supplemented with different combinations of plant growth regulators (PGRs), including BA, Thidiazuron (TDZ), NAA and Indole-3-butyric acid (IBA) (Table 1). Each culture medium also contained 7 g L^{-1} plant agar (Duchefa Biochemie, Italy), 30 g L^{-1} sucrose, and the pH was adjusted to 5.6–5.7 before autoclaving at 121°C for 20 min. Three microboxes, containing six MB slices each, were evaluated for each medium composition. Data on the MB regeneration frequency evaluated as the percentage of MB slices regenerating at least one shoot as well as the average number of regenerating shoots per explant were acquired after 30 days.

Table 1. Combination of basal salts and growth regulators used in the regeneration media

Culture media	Basal salts	Growth regulators (μM)			
		BAP	IBA	TDZ	NAA
QL1	QL	4.4	0.5		
QL2	QL	8.9	0.5		
QL3	QL	4.4		0.2	
QL4	QL	8.9		0.2	
QL5	QL	4.4			0.1
QL6	QL	8.9			0.1
DKW1	DKW	4.4	0.5		
DKW2	DKW	8.9	0.5		
DKW3	DKW	4.4		0.2	
DKW4	DKW	8.9		0.2	
DKW5	DKW	4.4			0.1
DKW6	DKW	8.9			0.1
WPMm1	WPMm	4.4	0.5		
WPMm2	WPMm	8.9	0.5		
WPMm3	WPMm	4.4		0.2	
WPMm4	WPMm	8.9		0.2	
WPMm5	WPMm	4.4			0.1
WPMm6	WPMm	8.9			0.1

QL, salts and vitamins of Quoirin and Lepoivre (1977); DKW, salts and vitamins of Driver and Kuniyuki (1984); WPMm, microsalts and vitamins of Murashige and Skoog (1962) and modified macrosalts of Lloyd and McCown (1980): 0.65 mM $CaCl_2$, 5.93 mM Ca(NO3) x 4H2O, 1.25 mM KH_2PO_4, 5.68 mM K_2SO_4, 1.50 mM $MgSO_4$, 5 mM NH_4NO_3.

2.2.2. Effect of different gelling agents on shoot regeneration efficiency and shoot hyperhydricity

MB slices obtained from 30-day-old MBs cultured on IM3 were placed on culture media containing two different commercial agar i.e., 7 g L^{-1} plant agar (Duchefa Biochemie, Italy), or 6 g L^{-1} plant agar S1000 (B&V, Italy), referred to as PA and B&V respectively in this study. These different gelling agents were combined with the components and culture condition of the media QL2, QL6 and WPMm5 described in Table 1. Data on the average number of regenerating shoots per explant and the frequency of vitrified shoots regenerating per MB slice were acquired after 30 days of culture. The two gelling agents were added at the concentration recommended by the manufacturers, and these produced culture media with similar compactness.

2.3. Genetic transformation

2.3.1. Gene construct and Agrobacterium tumefaciens strain

In this study, a hairpin gene construct was designed to silence PPV virus, and it contains the constitutive promoter 35S (543 base-long) of CaMV (Cauliflower Mosaic Virus). Each arm of the hairpin structure was 194 base-long sequence, which spans from base 134 to base 327 of the PPV polyprotein (accession number X16415). The two arms were joined by 115 base-long InLAX intron of the LAX1 gene of *Medicago truncatula*. The hairpin sequence was cloned firstly in the pbin19 binary vector (Bevan, 1984) and secondly sub-cloned in the pK7WG2 binary vector (Karimi

et al., 2002), which expresses the eGFP protein, and that confers resistance to kanamycin through NosPromoter-nptII-NosTerminator cassette. The gene construct (ihp35S-PPV194::eGFP) was inserted in *A. tumefaciens* strain EHA105 by electroporation (Sambrook et al., 1989).

2.3.2. Genetic transformation protocol and selection of GFP fluorescing calli

Transformation trial was carried out following the protocol previously described (Sabbadini et al., 2015) with some modifications. Single colonies of *A. tumefaciens* strain EHA105 were inoculated in liquid YEB medium supplemented with 152 μM spectynomicin and 61 μM rifampicin to reach an OD600 = 1.0–1.2. The bacterial culture was pelleted and re-suspended in MS basal salt and vitamins liquid medium including 20 g L^{-1} sucrose, 100 μM acetosyringone, pH 5.2. The explants were then dipped in the infection solution for 15 min, subsequently dried on sterile filter paper, and finally placed on co-culture medium (MS basal salt and vitamins, 30 g L^{-1} sucrose, 100 μM acetosyringone and 7 g L^{-1} plant agar) for 48 h at 24 ± 1 °C in dark conditions. After co-culture, MB slices were placed on regeneration medium WPMm5 supplemented with 0, 17 or 42 μM Kan, to select putative transgenic lines and/or calli. To control *A. tumefaciens* contaminations 473 μM carbenicillin plus 419 μM cefotaxime were used.

For each of the three treatments (Kan 0, 17 or 42 μM), 12 microboxes were prepared; each containing five MB slices, for a total of 60 MB slices per treatment. Data on the mean number of GFP spot per explant and the percentage of MB slices showing GFP fluorescing calli area were acquired at three and 12 weeks after co-culture. Furthermore, large coalesced zones (Lzs) of GFP expression (actively growing calli showing GFP fluorescence), were annotated as well as the frequency of MB slices showing Lzs of GFP expression at three and 12 weeks after co-culture. GFP fluorescing calli were detected by observing the explants under the stereomicroscope Leica MZ10F (Leica Mikrosystems GmbH, Wetzlar, Germany) (λEX = 480nm and λEM = 510 nm), and by using the Leica DFC 450 C290 camera for images acquisition, which were processed through Leica Application Suite V.4.5 (Leica Microsystems GmbH, Wetxlar, Germany).

2.4. Molecular analysis of GFP fluorescing calli

About 100 mg calli from four GFP fluorescing and one wild-type callus lines derived from different starting MBs of Hansen 536 were collected. Total genomic DNA was isolated from each callus line using the commercial kit "Nucleon Phytopure" (Amersham Bioscience) following the manufacturer instructions. PCR analyses were carried out to amplify 701 bp of the eGFP coding sequence with the following primers: eGFP-F, 50-GTGAGCAAGGGCGAGGAG-30 and eGFP-R, 50-TCCATGCCGAGAGTGATCCC-30. Furthermore, 456 bp of the ihp35S-PPV194 gene sequence, which spans from the antisense arm of the hairpin sequence to the NOS terminator (NOS ter), were amplified with the following primers: hpPPV-F, 50-TAGCTGTTGCACTCTCATATGTGTTT-30 and NOSTer-R, 50-GGAAGGGAC TGGCTGCTATTGGGCGAA-30. Total RNA was extracted from 100 mg of the collected samples with RNeasy® Plant mini Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. Samples were treated with DNase to be cleaned from any genomic DNA contamination, and 1 μg of total RNA was primed with oligo(dT)15 and reverse transcribed using GoscriptTM Reverse Transcription System (Promega) following the manufacturer instructions. For the expression analysis of the ihp35S-PPV194::eGFP gene construct, 701 bp of the eGFP coding sequence and 456 bp of the ihp35S-PPV194 gene sequence were amplified with the same pair of primers used for PCR analysis. A fragment of 129 bp of the translation elongation factor 2 (Tef2) coding sequence was amplified from each sample and used as positive control (Tong et al., 2009). The PCR conditions were as follows: 95°C

for 5 min; 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. The plasmid DNA of pK7WG2-ihp35S-PPV194::eGFP was used as positive control, while the DNA or cDNA from a wild-type callus line, and sample containing RNA template were used as negative controls. 10 µL of amplified fragments were loaded on agarose gel (2%, w/v) with SYBER® Safe DNA Gel Stain (Invitrogen) and detected by UV after electrophoresis.

4.5. Statistical analysis

The results obtained were analyzed by one-way ANOVA, and Duncan test ($p < 0.05$) was used to identify significant differences.

3. Results

3.1. Influence of basal media, growth regulators and gelling agents on regeneration efficiency of MBs

Eighteen combinations consisting of different basal media and plant growth regulators (PGRs) were applied to increase regeneration efficiency of Hansen 536 MBs. Generally, the results obtained showed higher regeneration efficiency, when Quoirin & Lepoivre medium (QL) and modified WPM (WPMm) were used as basal media (Table 2). Results also presented some optimal combinations, particularly media QL2, QL6 and WPMm5 showed higher values in terms of regeneration frequency, i.e., 83.3%, 83.3% and 77.8%, respectively. These combinations contained either QL or WPMm as basal media, enriched with BA combined with either NAA or Indole-3-butyric acid (IBA). On the contrary, except for medium DKW/Juglans six (DKW6), all PGRs combinations used with DKW as basal medium reported lower efficiency. A widespread problem of hyperhydricity was observed in the culture media tested, which were all supplemented with plant agar (PA) as gelling agent. Thus, a second regeneration trial was carried out with the main aim of reducing number of hyperhydrated shoots, by adding to media QL2, QL6 and WPMm5, 6 g L^{-1} B&V in comparison to the addition of 7 g L^{-1} PA. Results showed a significant increase in regeneration efficiency (mean number of regenerating shoots per explant) only in medium WPMm5 when MB slices were cultured on media with B&V instead of PA (Table 3). While, the frequency of vitrified shoots decreased significantly for all the three media combinations when explants were placed on media containing 6 g L^{-1} B&V; medium WPMm5 showed the best results. For this reason and considering the high regeneration efficiency obtained from the two trials, medium WPMm5 (modified WPM, 4.4 µM BA, 0.1 µM NAA, 6.0 g L^{-1} B&V agar) was chosen as regeneration medium for the transformation experiment in this study.

Table 2. Regeneration efficiency obtained from Hansen 536 MB slices after four weeks of culture on different combinations of basal media and PGRs

Regeneration media	Frequency of regeneration (%) ± SE [x]	Average number of regenerated shoots/MB ± SE
QL1	61.1 ± 5.5 (abc)	1.8 ± 0.5 (defg)
QL2	83.3 ± 9.6 (a)	6.4 ± 1.2 (ab)
QL3	61.1 ± 11.1 (abc)	3.8 ± 1 (bcde)
QL4	50 ± 0 (abc)	2.8 ± 1.9 (cdefg)
QL5	66.7 ± 9.6 (abc)	4.3 ± 1.2 (bcd)
QL6	83.3 ± 16.7 (a)	7.2 ± 1.3 (a)
DKW1	38.9 ± 5.5 (abc)	1.4 ± 0.6 (defg)
DKW2	38.9 ± 22.2 (abc)	1.3 ± 0.5 (defg)
DKW3	27.8 ± 5.5 (bc)	0.8 ± 0.3 (fg)
DKW4	22.2 ± 11.1 (c)	0.4 ± 0.2 (g)
DKW5	27.8 ± 5.5 (bc)	1.2 ± 0.5 (efg)
DKW6	72.2 ± 14.7 (ab)	3.5 ± 0.8 (bcdef)
WPMm1	61.1 ± 22.2 (abc)	2.6 ± 0.7 (cdefg)
WPMm2	72.2 ± 11.1 (ab)	5.5 ± 1.4 (abc)
WPMm3	38.9 ± 5.5 (abc)	1.4 ± 0.5 (defg)
WPMm4	72.2 ± 11.1 (ab)	3.3 ± 0.8 (cdefg)
WPMm5	77.8 ± 5.5 (a)	4.2 ± 0.9 (bcde)
WPMm6	50 ± 25.4 (abc)	2.1 ± 0.7 (defg)

[x] Number of MB slices with at least one shoot per total number of treated MB slices x 100. Values are means (n = 18) ± standard error (SE). Means in columns with different letters are significantly different according to Duncan test ($p < 0.05$).

Table 3. Regeneration efficiency and the percentage of vitrified shoots for MB slices cultured for four weeks on regeneration media containing different gelling agents

Gelling agent concentration (g L^{-1})		Average number of regenerated shoots/MB ± SE			Frequency of vitrified shoots (%) ± SE [x]		
		QL2	QL6	WPMm5	QL2	QL6	WPMm5
PA	7	$6.7 \pm 0.5^{(ab)}$	$7.4 \pm 1.3^{(ab)}$	$4.4 \pm 0.7^{(b)}$	$82.6 \pm 5.7^{(b)}$	$98.5 \pm 8.9^{(a)}$	$59.2 \pm 5.9^{(cd)}$
B&V	6	$5.6 \pm 1^{(ab)}$	$8.9 \pm 0.7^{(a)}$	$7.8 \pm 1.4^{(a)}$	$53.7 \pm 4.3^{(d)}$	$64.3 \pm 7.4^{(c)}$	$22.8 \pm 4.9^{(f)}$

PA, plant agar (Duchefa Biochemie, Italy); B&V, PAS1000, plant agar (B&V, Parma, Italy). * Number of vitrified shoots per total number of treated MB slices x 100. Values are means (n = 18) ± standard error (SE). Means with significant ($p < 0.05$) differences are indicated with different letters according to Duncan test.

3.2. Hansen 536 MBs transformation efficiency and the role of GFP and kanamycin as selectable markers

MB slices obtained from 30-day-old MBs cultured on medium WPMm5 were used as starting explants for *Agrobacterium*-mediated transformation trial (Figure 1). In a previous study carried out by us, we investigated the sensitivity of untransformed Hansen 536 MBs to kanamycin by placing the explants on WPMm5 medium with different kanamycin (Kan) concentrations (from 0 up to 117 µM); the results obtained showed that Kan concentrations higher than 50 µM extremely inhibited the *in vitro* regeneration of Hansen 536 untransformed MBs, hence leading to the complete necrosis of the explants after six weeks of culture (data not shown). Thus, lower concentrations of Kan (0, 17 or 42 µM) were tested in this study to allow the survival of putatively transformed cells and the eventual possibility of regenerating transgenic shoots. The influence of Kan on the frequency of GFP fluorescing explants was investigated. The number of Hansen 536 MB slices with at least one fluorescing spot and the presence of actively growing calli showing GFP were annotated at three and 12 weeks after co-culture on medium WPMm5 enriched with 0, 17 or 42 µM Kan (Table 4). The results obtained showed significant differences, related to the culture conditions applied (i.e., 0, 17 or 42 µM Kan), in terms of both percentage of MB slices expressing GFP and mean number of GFP spots per MB slice at three weeks after co-culture. The higher value (3.17) regarding the mean number of GFP spot/explant was recorded for the MB slices cultured on 42 µM Kan, while the highest value (about 63%) in terms of percentage of GFP fluorescing MB slices was obtained on 17 µM Kan (Table 4); in contrast, no statistical differences were observed at 12 weeks after co-culture. Results related to the percentage of MB slices presenting Large coalesced zones (Lzs) expressing GFP at each culture condition presented better results (about 37 %) when explants were placed on media with 42 µM Kan at three weeks after co-culture. In particular, a total number of 12, 15 and 22 MB slices (calli derived from different starting MBs) presenting Lzs of GFP expression were selected at 0, 17 and 42 µM Kan, respectively (Table 4 and Figure 2). However, at 12 weeks after co-culture, explants placed on 17 µM Kan showed higher values compared to the other two Kan concentrations. Indeed, about 6% of MB slices presented Lzs of GFP expression actively growing on 17 µM Kan (Table 4 and Figure 3 a, b), while 3% and 1% of MB slices with Lzs expressing GFP were obtained on 0 and 42 µM Kan, respectively (Table 4). In particular, the absence of kanamycin selection in the regeneration medium led to a major production of chimeric calli, in which the containment of non-transformed calli sections was challenging, and often the latter prevailed over the transformed ones over time; on the contrary, explants cultured in the presence of 42 µM Kan tended to necrotize during the 12 weeks after co-culture.

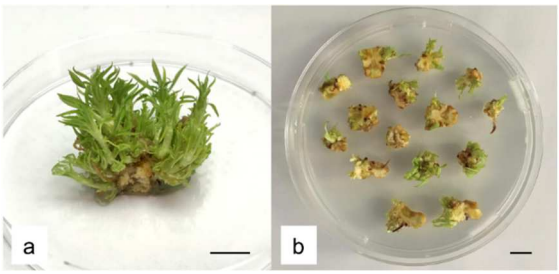

Figure 1. Hansen 536 regeneration via organogenesis: (**a**) Adventitious shoot regeneration after 30 days of culture on WPMm5 medium. (**b**) MB slices (1 cm2, 2 mm thick) used as starting explant for the transformation experiment (bar = 1 cm).

Table 4. GFP expression efficiency ofHansen 536MB slices after three and 12weeks of culture onWPMm5 medium supplemented with different kanamycin concentrations, or without kanamycin selection

Kan concentration (µM)	Number of weeks after co-culture	Mean number of GFP spot/ explant ± SE	GFP MB (%) ± SE[x]	Number of MB slices with Lzs	GFP MB Lzs (%) ± SE[y]
0	3	1.17 ± 0.37$^{(b)}$	26.67 ± 5.75$^{(c)}$	12	20 ± 5.21$^{(ns)}$
	12	0.82 ± 0.38$^{(A)}$	6.67 ± 3.25$^{(A)}$	2	3.33 ± 2.34$^{(NS)}$
17	3	2.10 ± 0.33$^{(ab)}$	63.33 ± 6.5$^{(a)}$	15	25 ± 5.64$^{(ns)}$
	12	0.78 ± 0.29$^{(A)}$	13.33 ± 4.42$^{(A)}$	4	6.67 ± 3.25$^{(NS)}$
42	3	3.17 ± 0.24$^{(a)}$	48.33 ± 6.27$^{(b)}$	22	36,67 ± 6.27$^{(ns)}$
	12	0.68 ± 0.36$^{(A)}$	8.33 ± 3.60$^{(A)}$	1	1.67 ± 1.67$^{(NS)}$

* GFP MB (%), Percentage of MB slices showing GFP fluorescence, selected either by kanamycin and GFP screening or by GFP visual selection alone (0 µM Kan), after three and 12 weeks on culture media. ** GFP MB Lzs (%), Percentage of MB slices showing large zones of GFP expression per total number of *Agrobacterium* inoculated MB slices. Values followed by small letters (a, b, c) compare explants cultured at three weeks after co-culture, while capital letter (A, B, C) compare explants cultured at 12 weeks after co-culture. Values are means (n = 60) ± standard error (SE). Means in columnswith different letters are significantly different according to Duncan test ($p < 0.05$). NS, not significant.

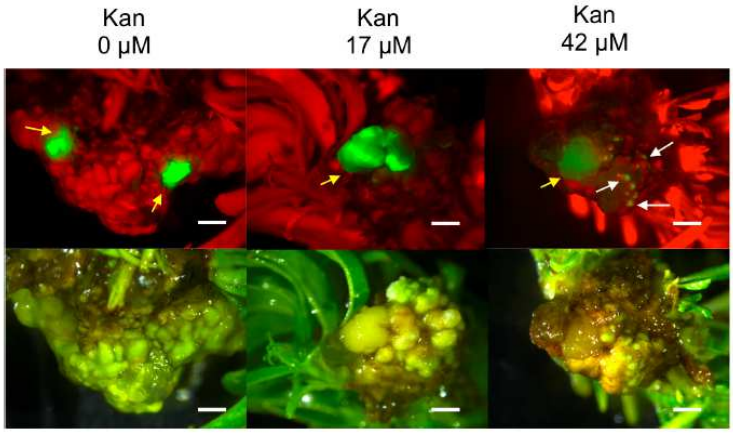

Figure 2. Hansen 536 GFP fluorescing spots and/or Lzs detected on MB slices at three weeks after co-culture on medium with 0, 17 or 42 µM Kan. White and yellow arrows indicate examples of GFP spots and large zones of fluorescent actively growing calli, respectively. Uniform bright green fluorescence was observed under UV light. Upper and lower panels show images taken under UV and white light, respectively (bar = 2 mm).

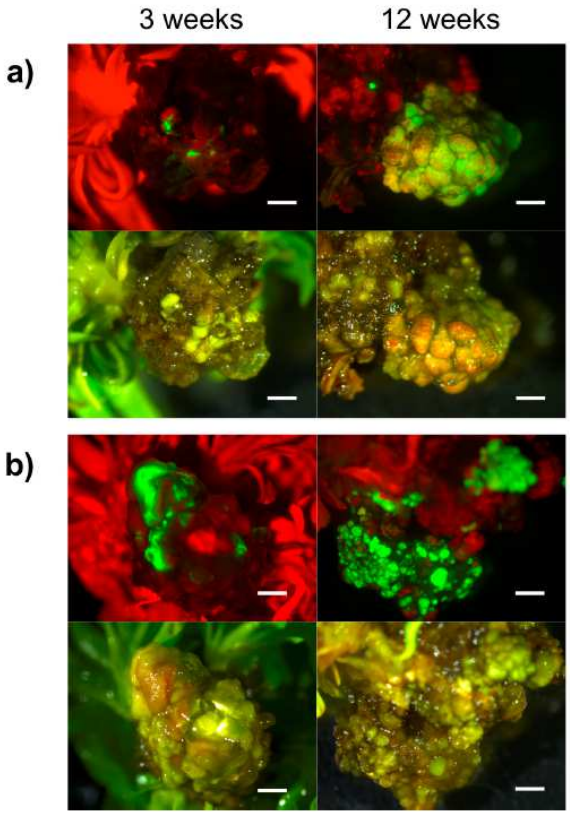

Figure 3. Hansen 536 GFP fluorescing spots and/or Lzs detected on MB slices at three weeks and 12 weeks after co-culture on medium with 17 µM Kan: (**a**, **b**) MB slices obtained from two independent MBs presenting Lzs of GFP expression actively growing within 12 weeks of culture. Uniform bright green fluorescence was observed under UV light. Upper and lower panels show images taken under UV and white light, respectively (bar = 2 mm).

3.3. Molecular analysis of putatively transgenic callus lines

Twelve weeks after co-culture, there were no transgenic shoots regenerated from Lzs detected, but stable GFP expressing callus lines (calli derived from different starting MBs) (Table 4) were isolated, divided into small pieces and placed on fresh WPMm5medium for proliferation with the corresponding Kan concentrations (i.e., 0, 17 or 42 µM Kan). One to three grams of calli were generated after two months of culture (with monthly sub-culture) from each GFP fluorescing callus line (Figure 4 a, b), which provided sufficient material for molecular analyses. Total genomic DNA and RNA were extracted from one wild-type callus line and four GFP expressing callus lines, two from those proliferating on 17 µM Kan, one proliferating on 42 µM Kan and one from those proliferating in 0 µM of Kan in the medium, respectively. The insertion and the transcription of eGFP gene and ihp35S-PPV194 sequence into the callus lines genomes were checked by PCR and RT-PCR analysis. 701 bp of the eGFP coding sequence and 456 bp of the ihp35S-PPV194 sequence were successfully amplified from the four GFP fluorescing callus lines analysed, confirming their transgenic state and the stable expression of the ihp35S-PPV194::eGFP gene construct at five months after *Agrobacterium* infection (Figure 4 c, d).

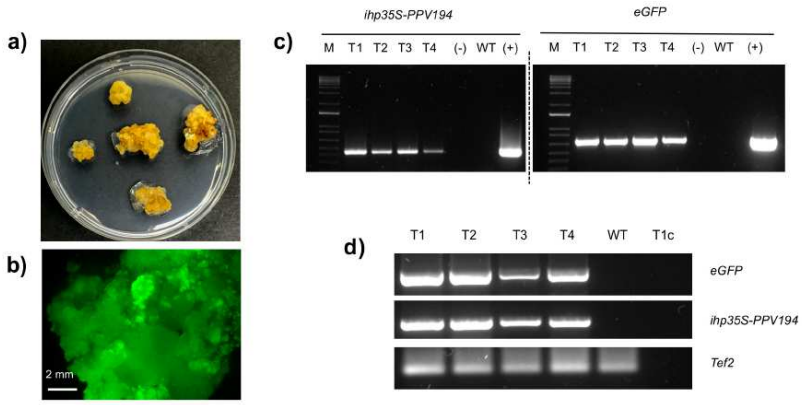

Figure 4. Hansen 536 transgenic callus lines and molecular analysis of transformants: (**a**) Callus line proliferated for five months after *Agrobacterium* infection. (**b**) Transgenic callus expressing uniform GFP fluorescence. (**c**) Amplification of ihp35S-PPV194 (456 bp) and eGFP (701 bp) genes fragments from the genomic DNAs of four transgenic (T1-T4) and one wild-type (WT) callus lines obtained from different MBs, and pK7WG2-ihp35S-PPV194::eGFP "(+)". The lane labelled "(-)" shows the PCR result using water as negative control. M, DNA marker (1Kb Plus DNA Ladder, Invitrogen, Carlsbad, CA, USA). (**d**) RT-PCR analysis of eGFP (701 bp), ihp35S-PPV194 (456 bp) sequences of four transgenic (T1-T4) and one wild-type (WT) callus lines obtained from different MBs. The 129 bp fragment of cDNA from housekeeping gene Tef2 was amplified as control to validate RT-PCR results. The lane labelled "T1c" shows the PCR result using total RNA of the line T1 as template control (RNA sample treated with DNase but without reverse transcription was used as PCR template to evaluate DNA contamination).

4. Discussion

There are few reports describing successful methods for the regeneration of adventitious shoots in peach when using adult tissues as starting plant material, and a few of them are focused on the use of MBs as efficient starting explant for shoot regeneration from both peach rootstocks (Garnem and GF677) and scions (UFO-3, Maruja, Flariba and Alice Bigi) (Girolomini et al., 2012; Pérez-Jiménez et al., 2012; Sabbadini et al., 2015). The MB has been described as a cluster of cells able to continuously regenerate new adventitious buds distinguishable at the surface of the callus (Mezzetti et al., 2002); histological studies showed that, internally, MB is composed of hypertrophied parenchymatic cells and initiation nodules from which the adventitious shoots originate (Mezzetti et al., 2002). Several studies confirmed the versatility of this regeneration protocol, also for the *in vitro* organogenesis of plant species other than peach (Cappelletti et al., 2016; Sabbadini et al., 2019; Xie et al., 2016); however, its optimization is required for the individual genotypes under study, that often showed different levels of reaction to external induction treatments in regards to *in vitro* morphogenesis (Pérez-Jiménez et al., 2012; Xie et al., 2016). It has been reported that several of the differences observed in the organogenetic response, even within genotypes of the same species, greatly depend on the level of reaction to PGRs signals, which can vary also depending to the starting explant used (Declerck and Korban, 1996; Jiménez, 2005; Pérez-Jiménez et al., 2014). The use of different basal salts tested in our study, proved that QL and WPMm were more appropriate combinations of micro and macro salts for regeneration of Hansen 536 MB slices. In particular, our investigation showed a positive effect on shoots regeneration of Hansen 536 when QL or WPMm were supplemented with the cytokinin BA combined either with NAA or IBA; similar results were reported by other authors working on organogenesis of *Prunus* spp. and also of other fruit species (Abdollahi et al., 2006; Escalettes and Dosba, 1993; Gentile et al., 2002; Pérez-Jiménez et al., 2012, 2013; Pérez-Tornero et al., 2000; Petri and Scorza, 2010; Tang et al., 2002). The effect of gelling agents on Hansen 536 MB slices was also investigated. The strong influence that composition and concentration of gelling agents have in regard to shoot hyperhydricity and plant regeneration efficiency in woody fruit tree species has been well documented (Burgos and Alburquerque, 2003; Chevreau et al., 1997; Debergh, 1983; Pérez-Tornero et al., 2000). Our results indicated that, the use of B&V as the gelling agent had a

positive effect on Hansen 536 regeneration, as well as reduction of hyperhydrated shoot percentage, compared to PA agar. Even though, the composition and gel strength of the two agars used are quite similar, they led to significantly different results, especially concerning hyperhydricity effect on Hansen 536 shoots. Furthermore, the combination of B&V agar with medium WPMm5 induced a significant decrease in the percentage of hyperhydrated shoots compared to the other basal media and PGRs combinations tested (media QL2 and QL6) (Table 3). This revealed that, several factors other than gelling agents have a role on hyperhydricity and shoot regeneration. Several studies have reported on the role of single ions present in culture media, such as NH_4, NO_3^- and Ca^{2+}, on *in vitro* plant culture in relation with hyperhydricity (Alanagh et al., 2014; Brand, 1993; Ivanova and van Staden, 2009). In particular, Alanagh et al. (2014), found that a low concentration of Ca^{2+} (lower than 3 mM) combined with a medium concentration of NO_3^- (between 7–21 mM) is recommended for the proliferation of healthy shoots of peach rootstock GF677. Medium WPMm5 used in our investigation contained 16.86 mM of NO_3^- combined to 6.6 mM of Ca^{2+}, in comparison with medium QL2 and QL6, which are characterized by a lower Ca^{2+} concentration (3.53 mM) with almost double content of total NO_3^- (29.88 mM). This higher content of NO_3^- could explain the higher number of hyperhydrated shoots obtained in our study when MB slices were cultured on media QL2 and QL6.

The optimized regeneration medium was utilized for subsequent genetic transformation trial with the hairpin gene construct ihp35S-PPV194, which also achieved selection of putative transgenic tissues through the two marker genes, eGFP and nptII. A similar hairpin sequence was already validated in a previous study carried out in *N. benthamiana* plants, demonstrating its ability in conferring systemic resistance against PPV virus (Pandolfini et al., 2003). With the aim to achieve stable transformation with this construct, GFP expression on proliferating cells after *Agrobacterium* infection of Hansen 536 MB slices was exploited to isolate and proliferate stable transgenic callus lines. In this regard, expression of the vital reporter gene eGFP was crucial to assess the progress of GFP fluorescing calli formation in the first 12 weeks after co-culture. Furthermore, it helped in evaluating the possible role of kanamycin in stabilizing transient expression in cells at three weeks after co-culture; most transgenic events are considered stable after this period (Padilla et al., 2006; Tee et al., 2003). The results obtained showed how the transformation protocol used, previously applied on GF677 MB slices (Sabbadini et al., 2015), was also effective for the production of Hansen 536 GFP fluorescing actively growing calli expressing the ihp35S-PPV194::eGFP construct in all the three culture conditions applied (0, 17, 42 µM Kan), with best results obtained when 17 µM Kan was supplemented in the culture medium (Figure 3 and Table 4). In addition, the combined use of GFP and kanamycin selection facilitated the early elimination of regenerating escapes (both shoots and calli sectors), which compete against transformed cells. Similar results have been reported in *Prunus* spp., including peach, and other woody plant species genetic transformation, confirming the usefulness of reporter genes and antibiotic selection in increasing the frequency of transgenic events and avoiding regeneration of escapes (Ghorbel et al., 1999; Padilla et al., 2003; Padilla et al., 2006; Xie et al., 2016; Yau et al., 2008). In this study, the selection of transgenic events, even in absence of kanamycin in the medium, was investigated; in this culture condition about 20% (at three weeks) and 3% (at 12 weeks) of MB slices with large coalesced zones of GFP expression were selected using GFP screening alone (Figure 2 and Table 4). We recently demonstrated in a similar work performed on grapevine MBs, the possibility of isolating transgenic shoots/calli lines with only the use of non-destructive marker genes, like gfp (Sabbadini et al., 2019). However, similarly to the results obtained in the present study, we observed a lower effciency, both in terms of frequency of transgenic plants/calli lines obtained and proliferation ability of GFP expressing cells, when MB slices were cultured on non-selective conditions (Sabbadini et al., 2019). Indeed, in the present study the results obtained confirm the strong influence played by kanamycin in obtaining transgenic events in a more effective way, especially during the first weeks of selection (Table

4), inhibiting also the regeneration of non-transformed shoots (escapes) or the proliferation of non-transgenic calli sectors. Nevertheless, the obtainment of transgenic actively growing calli of Hansen 536 also in the absence of kanamycin in the medium at early stages of transformation, open the possibility, even though with a much lower frequency, of isolating transgenic calli exploiting only reporter genes as selective agent. This scenario would help to avoid the use of antibiotic-based selection strategies to obtain antibiotic marker-free transformed plants, reducing the risk assessment process and public concerns (Ballester et al., 2008; Breyer et al., 2014; Rosellini, 2012).

In conclusion, we established an efficient regeneration protocol to obtain adventitious shoots of Hansen 536 peach rootstock starting from somatic tissues; this protocol was exploited for subsequent transformation trial that led to the recovery of stable transgenic callus lines of Hansen 536 stably expressing the ihp35S-PPV194::eGFP gene construct. Even if the protocol needs to be optimized for inducing shoot regeneration from these transgenic callus lines, to our knowledge this represents one of the few examples, or even the first successful attempt of producing transformed peach calli, and a promising protocol to obtain regenerated shoots expressing the anti-PPV RNAi construct from peach somatic tissue. Furthermore, these *in vitro* proliferating transgenic calli represent a valuable tool to both explore the genetic and molecular features that hamper the regeneration of transgenic shoots and also for future functional analyses of genes and omics studies (Ainsley et al., 2001; Zhao et al., 2017).

[1]This chapter has been published: Sabbadini, S., **Ricci, A.**, Limera, C., Baldoni, D., Capriotti, L., Mezzetti, B. (2019) Factors affecting the regeneration, via organogenesis, and the selection of transgenic calli in the peach rootstock Hansen 536 (*Prunus persica*× *Prunus amygdalus*) to express an RNAi construct against PPV virus. *Plants*, *8*(6), 178. DOI: 10.3390/plants8060178

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CHAPTER 4

RESEARCH SECTION

ADVENTITIOUS SHOOT REGENERATION FROM *IN VITRO* LEAF EXPLANTS OF THE PEACH ROOTSTOCK HANSEN 536[1]

1. Introduction

The genetic improvement of woody fruit species, including peach, through biotechnological approaches is often limited by the lack of tissue culture systems that allow the delivery of desired traits in the plant host genome and by the subsequent regeneration of the selected transgenic lines. Thus, the establishment of efficient *in vitro* adventitious regeneration methods represents a prerequisite for the development and application of effective transformation procedures aimed to genetically improve perennial crops (Xu et al., 2020).

Among fruit trees, peach is recognized as one of the most recalcitrant species in terms of *in vitro* organogenesis (Pérez-Jiménez et al., 2012), and only a few studies report the establishment of efficient and reproducible regeneration protocols starting from different explants of peach rootstocks and cultivars. Although the use of adult tissues is highly recommended to preserve desirable traits of peach selected clones, adventitious shoots regeneration starting from seed-derived material has been the main successful approach used in the past for this species (Bhansali et al., 1990; Hammerschlag et al., 1985; Pérez-Clemente et al., 2004; Scorza et al., 1990; Smigocki and Hammerschlag, 1991). However, peach adventitious shoot regeneration, avoiding the use of juvenile tissues, has also been recorded starting from meristematic tissues (Pérez-Jiménez et al., 2012; Sabbadini et al., 2019; Sabbadini et al., 2015) and leaves (Gentile et al., 2002; San et al., 2014; Zhou et al., 2010; Zong et al., 2019). In particular, Gentile et al. (2002) described the only study reporting *in vitro* shoot organogenesis from leaves of *Prunus persica* L. cultivars (from expanded leaves, from leaves of preconditioned shoot apices and from leaves of adventitious shoots) (Gentile et al., 2002). Other results showing adventitious shoot regeneration from leaves have been reported for the peach hybrid rootstocks Nemaguard (*P. persica x P. davidiana*) (Zhou et al., 2010), Guardian® (BY520-9 peach seedling rootstock) (San et al., 2014) and Hansen 536 (*P. persica x P. amygdalus*) (Zong et al., 2019). These reports show the difficulty in setting up a versatile and efficient protocol for peach leaf organogenesis, which often depends on the genotype selected, and it is highly influenced by the starting explant used, by the macro-/microelements and plant growth regulators (PGRs) composing the basal culture medium and by the exposure to different hours/weeks of darkness/light during explant *in vitro* cultures. Furthermore, several studies describe how the accumulation of ethylene during *in vitro* tissue culture can have a negative impact on shoot regeneration (reviewed by Biddington, 1992). Silver thiosulfate (STS), an ethylene inhibitor (Veen and van de Geijn, 1978), has been successfully utilized as the promoter of adventitious shoot regeneration from leaf tissues of *P. armenica* L. (Burgos and Alburquerque, 2003), *P. serotina* Ehrh. (Liu and Pijut, 2008) and *P. domestica* L. (Petri and Scorza, 2010). The only report investigating the effect of STS on shoot regeneration from peach leaf explants is reported by San et al., (2015), where a regeneration rate of 16% of the peach rootstock Guardian® was described.

The development of efficient protocols for shoot regeneration starting from somatic tissues of peach rootstocks is one of the primary objectives required for the application of gene-delivery technologies to this recalcitrant fruit crop (Byrne et al., 2012; San et al., 2015; Zhou et al., 2010; Zong et al., 2019). In fact, peach rootstocks, such as peach x almond hybrids, have been extensively used for the propagation of peach plants, given their favourable agronomic

traits, such as high tolerance to calcareous and dry soils, good productivity induced in the grafted peach scion, and resistance to several plant pathogens and pests (Byrne et al., 2012; Connell et al., 2002). Among peach x almond hybrid rootstocks, Hansen 536 (*P. persica x P. amygdalus*) has been used for grafting both peach and almond genotypes and has reached a significant commercial importance especially in California (Zong et al., 2019). It is characterized by a high plant vigour also in poor and alkaline soils and shows resistance against some root-knot nematodes (*M. arenaria*, *M. incognita* and *M. javanica*) (Byrne et al., 2012; Felipe et al., 1997; Kester and Asay, 1986). However, this genotype needs further genetic improvement especially due to its low waterlogging tolerance, which prevents its spread also in other geographical area (Byrne et al., 2012; Zong et al., 2019).

In chapter 3, the optimization of an *in vitro* regeneration and transformation protocol for the peach hybrid rootstock Hansen 536 (*P. persica x P. amygdalus*) using meristematic bulks as starting explants was described. Even though the regeneration rate obtained through this system was about 80%, only transgenic callus lines were obtained that did not lead to the regeneration of any transgenic plant. One possible solution to this bottleneck can be the development and use of a different regeneration protocol from somatic tissues, more suitable to obtain Hansen 536 transgenic plants in future transformation trials. The present study aimed to establish the optimal conditions necessary to induce an efficient organogenetic response from *in vitro* Hansen 536 leaf explants by evaluating the effect of different PGRs concentrations and combinations added to the basal regeneration medium. Furthermore, the impact of different compounds that could significantly enhance adventitious shoot regeneration efficiency was evaluated. In particular, we studied the influence of STS on Hansen 536 shoot regeneration as well as the effect on the regeneration efficiency of different antibiotics, which are often used in genetic transformation systems to contain *Agrobacterium* overgrowth. The established protocol represents a useful tool for the optimization of new genetic transformation methods and for the application of biotechnological approaches aimed at improving genetically Hansen 536 and other peach rootstocks and varieties.

2. Materials and methods

2.1. Establishment of in vitro shoots

Shoot tips (approximately 0.5 cm long) were cut from 10-cm-long shoots of 10-year-old greenhouse-grown peach x almond hybrid rootstock Hansen 536 (*P. persica x P. amygdalus*) trees at Vitroplant Italia, Cesena, Italy and used to establish peach *in vitro* cultures. The collected shoot tips were surface sterilized by washing them in 1% (V/V) sodium hypochlorite solution for 15 min, followed by three washes with sterile distilled water. Explants were then placed on shoot multiplication medium composed of McCown Woody Plant Medium (WPM) (Duchefa Biochemie, Haarlem, The Netherlands) basal salts and vitamins (Lloyd and McCown, 1980), 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000 (B&V, Reggio Emilia, Italy) supplemented with 6.6 µM N6-benzylaminopurine (BAP) (Duchefa Biochemie, Haarlem, The Netherlands) and 0.1 µM α–naphtalene acetic acid (NAA) (Duchefa Biochemie, Haarlem, The Netherlands). The final pH value was adjusted to 5.7 with KOH before autoclaving at 121°C for 20 min. *In vitro* cultures were kept in a growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol/m^2/s) provided by white fluorescent tubes, and they were periodically subcultured (2-week intervals) on fresh shoot multiplication medium for a total of three subcultures. Proliferating shoots were then transferred to the elongation medium in order to provide expanded leaves for this study. Preparation and composition of the elongation medium were the same as for the shoot multiplication medium, except for plant growth regulators (PGRs) concentrations, corresponding to 0.45 µM BAP and 0.1 µM NAA. *In vitro* shoots were maintained on the elongation medium for 20 days at the same light and temperature conditions as described above and then used for shoot regeneration trials.

2.2. General approach for adventitious shoot regeneration

The first four apical expanding leaves (about 1.5–2 cm in length) along with their petiole from 3-week-old elongated shoot cultures were used as starting explants in this study (Figure 1 a). The abaxial surface of each leaf was wounded about four times at each side perpendicular to the leaf mid vein, without fully separating the sections, and then placed with the abaxial side in contact with the regeneration medium (Figure 1 b, c). Leaves were cultured on WPM basal salts and vitamins, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000 (B&V, Reggio Emilia, Italy); the pH was adjusted to 5.7 before autoclaving at 121°C for 20 min, and then 25 mL of medium was poured into sterile plastic Petri dishes (9 cm x 1.5 cm). After leaves were placed on medium, the dishes were maintained in darkness at 24 ± 1°C for three weeks. *In vitro* leaves were then transferred to fresh media and exposed to light (16-h photoperiod at a light intensity of 40 µmol/m2/s) at 24 ± 1°C. Data on the leaf regeneration frequency and on the average of regenerating shoots per leaf were collected after five weeks from the beginning of the trial.

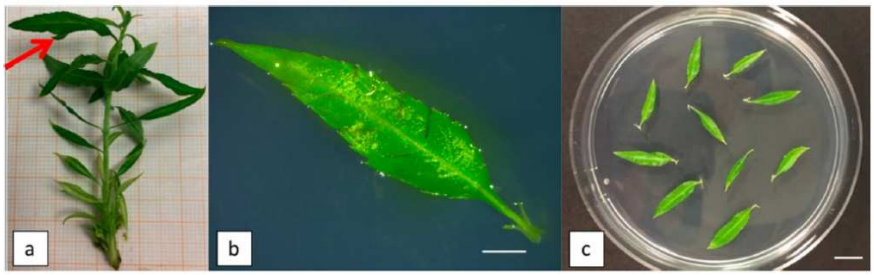


Figure 1. Plant material used as the starting explants in this study: (**a**) leaves collected from 3-week-old elongated shoots of Hansen 536. The arrow indicates the type of leaf collected and used in all the regeneration experiments; (**b**) the young leaf of the Hansen 536 wounded perpendicular to the mid vein and placed with the abaxial side on regeneration medium (bar = 2 mm), and (**c**) *in vitro* leaves of Hansen 536 placed on regeneration medium (bar = 1 cm).

2.3. Effect of different combinations and concentrations of PGRs

In this experiment, leaves were placed on regeneration media supplemented with cytokinins, as kinetin (KIN) (Duchefa Biochemie, Haarlem, The Netherlands) (14, 16.3 or 19 µM), BAP (11, 13.3 or 15.5 µM) or thidiazuron (TDZ) (Duchefa Biochemie, Haarlem, The Netherlands) (2.25, 4.5 or 9 µM) and with auxin, as NAA (0.25 µM), for a total of 20 different media combinations (Table 1).

Table 1. Different concentrations and combinations of PGRs used in the regeneration medium

Regeneration media	Plant Growth Regulators (µM)			
	KIN	BAP	TDZ	NAA
WPM 1	-	-	-	-
WPM 2	-	-	-	0.25
WPM 3	14	-	-	-
WPM 4	16.3	-	-	-
WPM 5	19	-	-	-
WPM 6	14	-	-	0.25
WPM 7	16.3	-	-	0.25
WPM 8	19	-	-	0.25
WPM 9	-	11	-	-
WPM 10	-	13.3	-	-
WPM 11	-	15.5	-	-
WPM 12	-	11	-	0.25
WPM 13	-	13.3	-	0.25
WPM 14	-	15.5	-	0.25
WPM 15	-	-	2.25	-
WPM 16	-	-	4.5	-
WPM 17	-	-	9	-
WPM 18	-	-	2.25	0.25
WPM 19	-	-	4.5	0.25
WPM 20	-	-	9	0.25

KIN, kinetin; BAP, N6-benzylaminopurine; TDZ, thidiazuron; NAA, α-naphtalene acetic acid.

2.4. Effect of silver thiosulphate

Young expanding leaves were placed on WPM 11 regeneration medium supplemented with different concentrations of silver thiosulphate (STS) (0, 10, 20, 40 or 80 µM). Preparation of media and culture conditions of the leaves were the same as previously described. Stock solutions (0.1 M) of sodium thiosulphate and silver nitrate were prepared by dissolving 790 mg of sodium thiosulphate (Sigma-Aldrich, Milan, Italy) and 850 mg of silver nitrate (Sigma-Aldrich, Milan, Italy) into 50 mL of ultrapure water. STS stock solution (0.02 M) was then prepared just before use by slowly adding 20 mL of silver nitrate stock solution (0.1 M) into 80 mL of sodium thiosulphate stock solution (0.1 M) (1:4 ratio). STS stock solution was filter-sterilised and added to the medium after autoclaving and cooling down to 50°C.

2.5. Effect of antibiotics

The influence of different antibiotics added to the WPM 11 medium, alone or combined (Table 3), on the regeneration of Hansen 536 leaves was evaluated using the same regeneration protocol previously reported. Filter-sterilised antibiotics were added to the regeneration medium after autoclaving and cooling down to 50°C.

2.6. Shoot elongation, rooting and acclimatization

Shoots (1 cm in length) regenerated from leaf explants in the above experiments were excised and propagated on WPM medium, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000 (B&V, Reggio Emilia, Italy) supplemented with 6.6 µM BAP and 0.1 µM NAA for a total of three subcultures (2-week intervals). Single shoots were then placed on WPM medium supplemented with 0.5 µM BAP and 7.38 µM indole-3-butyric acid (IBA) (Duchefa Biochemie, Haarlem, The Netherlands) for 20 days to induce rooting. Shoot cultures were placed in the growth chamber under a photoperiod of 16-h light (70 µmol/m^2/s) provided by white fluorescent tubes. *In vitro* rooted shoots were finally acclimatized in pots (7 x 7 cm) containing commercial peat and grown in the greenhouse.

2.7. Statistical analysis

For each treatment included in the regeneration experiments described above, five petri dishes were prepared and a total of fifty explants were used (ten leaves per dish). Three independent experiments were carried out for each regeneration trial. Regeneration frequency is expressed as the percentage of explants regenerating at least one shoot per total explants treated. Data on regeneration percentages were transformed by the arcsine square root transformation, ARSIN (SQRT (X)), before analysis. Shoot numbers are shown as the mean ± SE of the total number of shoots regenerating from starting leaves.

The results acquired were analyzed by one-way ANOVA using Statistica 7 software (Statsoft Tulsa, CA, USA), and means were separated using the Newman–Keuls test ($p < 0.05$).

3. Results

3.1. Influence of different combinations and concentrations of PGRs on regeneration efficiency from leaf explants

Twenty regeneration media (WPM 1–WPM 20) supplemented with different concentrations and combinations of PGRs were tested to evaluate the organogenetic competence and adventitious shoot regeneration efficiency of Hansen 536 leaf explants (Table 2). Three weeks after incubation in dark condition, calli appeared on petioles and formed around the cuts made perpendicular to the leaf mid vein, and some buds and adventitious shoots started to regenerate, mainly on the petiole surface. After five weeks of culture, about 100% caulogenesis was observed on leaves

cultured on most of the tested regeneration media (except for WPM 1, where no caulogenesis was recorded) (data not shown). Differences were observed for callus size based on the PGR combination used (Supplementary Figure S1). Data acquired after five weeks from the beginning of the trial revealed higher values of both regeneration frequency and average number of regenerating shoots per leaf when the explants were cultured on basal medium supplemented with different concentrations of N6-benzylaminopurine (BAP) used alone or in combination with 0.25 µM α-naphtalene acetic acid (NAA) (Table 2). In particular, the highest regeneration frequency and mean number of regenerating shoots per leaf, 53% and 0.77 ± 0.08, respectively, were obtained from explants cultured on medium containing 15.5 µM BAP alone (WPM 11), whereas the analysis of results obtained by using different cytokinins other than BAP in the basal medium, like kinetin (KIN) or thidiazuron (TDZ), showed a significant decrease in the organogenetic response of leaf explants, with regeneration rates up to 13% and 28% for WPM 6 and WPM 15, respectively. Furthermore, the combined use of NAA together with TDZ in the media seemed to have a negative influence on adventitious shoot regeneration compared to the use of TDZ alone (Table 2).

Table 2. Influence of plant growth regulators (PGRs) on the regeneration efficiency from Hansen 536 leaf (LF) explants cultured for five weeks on regeneration media

Regeneration media	Frequency of regeneration (%) ± SE*	Average number of regenerating shoots/LF ± SE
WPM 1	0 $^{(g)}$	0 $^{(e)}$
WPM 2	0 $^{(g)}$	0 $^{(e)}$
WPM 3	8.7 ± 2.3 $^{(fg)}$	0.12 ± 0.03 $^{(de)}$
WPM 4	9.3 ± 2.38 $^{(fg)}$	0.11 ± 0.04 $^{(de)}$
WPM 5	6.7 ± 2.04 $^{(fg)}$	0.07 ± 0.02 $^{(de)}$
WPM 6	13.3 ± 2.78 $^{(defg)}$	0.15 ± 0.03 $^{(cde)}$
WPM 7	3.3 ± 1.47 $^{(g)}$	0.03 ± 0.0.1 $^{(e)}$
WPM 8	2 ± 1.15 $^{(g)}$	0.02 ± 0.01 $^{(e)}$
WPM 9	26.7 ± 3.62 $^{(bcd)}$	0.41 ± 0.06 $^{(b)}$
WPM 10	24.7 ± 3.53 $^{(bcde)}$	0.39 ± 0.06 $^{(b)}$
WPM 11	53 ± 4.02 $^{(a)}$	0.77 ± 0.08 $^{(a)}$
WPM 12	29.3 ± 3.73 $^{(bc)}$	0.47 ± 0.07 $^{(b)}$
WPM 13	32 ± 3.82 $^{(bc)}$	0.39 ± 0.05 $^{(b)}$
WPM 14	37 ± 3.84 $^{(b)}$	0.48 ± 0.06 $^{(b)}$
WPM 15	28 ± 3.88 $^{(bc)}$	0.48 ± 0.06 $^{(b)}$
WPM 16	20 ± 3.59 $^{(cdef)}$	0.3 ± 0.06 $^{(bcd)}$
WPM 17	24 ± 3.49 $^{(bcde)}$	0.36 ± 0.07 $^{(bc)}$
WPM 18	12 ± 3.19 $^{(efg)}$	0.16 ± 0.05 $^{(de)}$
WPM 19	2 ± 2.72 $^{(g)}$	0.02 ± 0.04 $^{(e)}$
WPM 20	6 ± 2.04 $^{(fg)}$	0.06 ± 0.03 $^{(de)}$

* Number of explants regenerating at least one shoot per total explants treated x 100. One-way ANOVA was used to analyse the results. Different letters in the same column show significant differences at $p < 0.05$ by the Newman–Keuls test ± SE (n = 150). Each value represents the mean ± SE of three independent experiments. WPM, McCown Woody Plant Medium.

3.2. Influence of silver thiosulphate on regeneration efficiency from leaf explants

The effect of silver thiosulphate (STS) supplemented in WPM 11 medium on shoot organogenesis from Hansen 536 leaf explants was evaluated after five weeks of culture (Figure 2 a, b). STS did not increase explant regeneration frequency at any of the concentrations tested. The highest value (50%) was obtained by adding 10 µM STS to the regeneration medium, and it was not significantly different to the regeneration frequency observed from leaves placed on regeneration medium without STS (47%) (Figure 2 a), whereas the average number of regenerating shoots per leaf increased two-fold (a mean of 1.14 ± 0.13 shoots per explant) when STS was used at a concentration of 10 µM compared to the control (a mean of 0.61 ± 0.08 shoots per explant) (Figure 2 b – d). Concentrations of STS higher than 10 µM inhibited adventitious shoot regeneration progressively; in particular, when STS was used at 20, 40 or 80 µM, the regeneration frequency decreased drastically to 29.3%, 20% and 5.3%, respectively (Figure 2 a), while the average number of regenerating shoots per leaf decreased significantly by using a concentration of STS equal or higher than 40

µM (Figure 2 b). Furthermore, when STS was added to the regeneration medium at any concentration tested, we observed a higher frequency of shoots that also regenerated on the cuts made perpendicular to the leaf mid vein (Figure 2 e), while most of the shoots regenerated only on the petiole surface when explants were cultured without STS (data not shown).

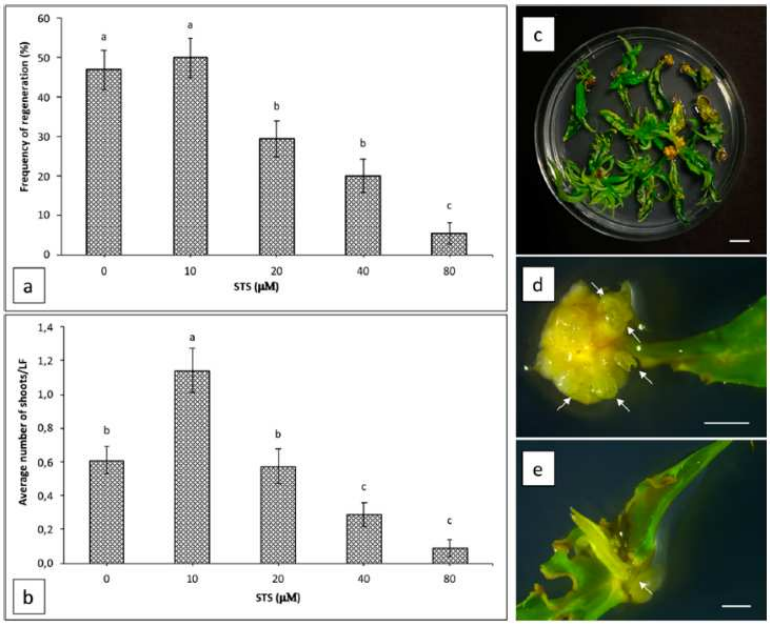


Figure 2. Regeneration efficiency of Hansen 536 leaf explants cultured on WPM11 supplemented with different concentrations of silver thiosulphate (STS): **(a)** frequency of regeneration expressed as the number of explants regenerating at least one shoot per total explants treated x 100 and **(b)** the average number of regenerating shoots per leaf (LF) after five weeks of culture. One-way ANOVA was used to analyse the results. Different letters show significant differences at $p < 0.05$ by Newman-Keuls test ± SE (n = 150). Each value represents the mean ± SE of three independent experiments. **(c)** Adventitious shoots regenerating from leaf explants after seven weeks of culture on WPM11 supplemented with 10 mM STS (bar = 1 cm). Adventitious shoots regenerating from petiole **(d)** and from cuts perpendicular to the leaf mid vein **(e)** of Hansen 536 leaves after three weeks of culture on WPM 11 supplemented with 10 mM STS (bar = 2 mm). White arrows indicate regenerating adventitious shoots.

3.3. Influence of antibiotics on regeneration efficiency from leaf explants

The effect of different antibiotics added to WPM 11 medium on adventitious shoot regeneration efficiency of Hansen 536 leaves was observed after five weeks of culture (Table 3). Similar to the results obtained when STS was supplemented in the basal regeneration medium, the addition of antibiotics also did not significantly increase the regeneration frequency of leaf explants compared to the control. However, the addition of carbenicillin (238 µM) plus cefotaxime (210 µM) in the WPM 11 medium positively affected the average number of regenerating shoots per leaf compared to the control and to the other antibiotics tested (Table 3). On the contrary, when the same antibiotics were applied alone at higher concentrations, regeneration rates decreased more than two-fold.

Table 3. Effect of antibiotics on the regeneration efficiency from Hansen 536 leaf (LF) explants cultured for five weeks on WPM 11 medium

Antibiotic(s)	mM	Frequency of regeneration (%) ± SE*	Average number of regenerating shoots/LF ± SE
None	0	49 ± 5.02 [(a)]	0.61 ± 0.08 [(b)]
Carbenicillin	475	19.3 ± 3.68 [(b)]	0.4 ± 0.07 [(bc)]
Cefotaxime	420	21.4 ± 3.86 [(b)]	0.33 ± 0.06 [(bc)]
Timentin	514	9.3 ± 1.41 [(b)]	0.17 ± 0.01 [(c)]
Carbenicillin/Cefotaxime	238/210	43 ± 4.94 [(a)]	0.91 ± 0.13 [(a)]
Timentin/Cefotaxime	171/210	21.3 ± 3.94 [(b)]	0.39 ± 0.07 [(bc)]

[w] Duchefa Biochemie, Haarlem, The Netherlands. [x] 15:1 mixture of ticarcillin and clavulanic acid. [y] Common concentrations found in the literature to control *Agrobacterium* growth. [z] Number of explants regenerating at least one shoot per total explants treated x 100. One-way ANOVA was used to analyse the results. Different letters in the same column show significant differences at $p < 0.05$ by the Newman–Keuls test ± SE (n = 150). Each value represents the mean ± SE of three independent experiments.

3.4. In vitro rooting and acclimatization of adventitious shoots

Adventitious shoots developed in the above treatments were *in vitro* elongated, rooted, and acclimatized. About 85% of them were able to produce *in vitro* roots (about 0.5–1 cm long) after 20 days in the rooting medium (Figure 3 a, b), and about 90% of them were successfully acclimatized to the greenhouse (Figure 3 c).

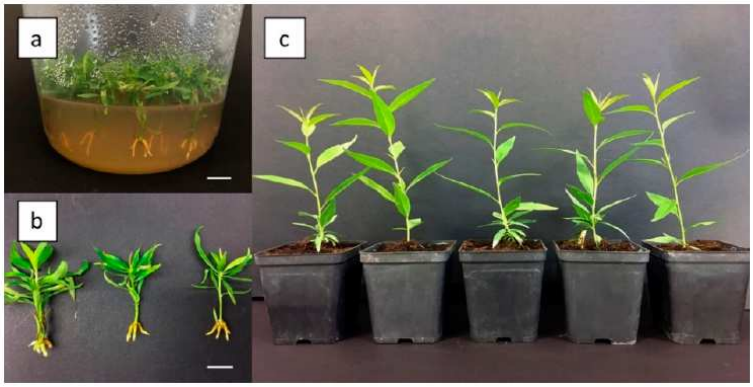


Figure 3. *In vitro* rooting and acclimatization of adventitious shoots from Hansen 536 leaves: **(a, b)** elongated and rooted *in vitro* adventitious shoots ready for acclimatization and **(c)** acclimatized rooted shoots in 7 × 7 cm pots.

4. Discussion

Efficient adventitious shoot regeneration, rooting, and acclimatization were achieved in this study by using leaves of the peach x almond hybrid rootstock Hansen 536 as the starting plant material. One of the most important factors affecting plant tissue regeneration is the type and concentrations of PGRs added to the regeneration medium. In this study, the highest regeneration efficiency (53%, with mean number of shoots per explant of 0.77 ± 0.08) was obtained when WPM basal medium was enriched with 15.5 µM BAP (WPM 11), albeit good regeneration rates (from 29.3% up to 37%) were also obtained when explants were placed on media supplemented with BAP in combination with NAA. Similarly, Gentile et al. (2002), reporting *in vitro* shoot induction from *P. persica* leaves, showed that the best results were obtained from leaves when BAP was used as cytokinin in the media; the highest shoot regeneration rate of 28.3% was recorded in peach cv. 842 Standard. Furthermore, Zong et al. (2019) have recently optimized a protocol for Hansen 536 *in vitro* leaf regeneration, which led to a maximum shoot regeneration rate of 36.1% when explants were cultured on WPM media supplemented with BAP in combination with indole-3-butyric acid IBA, confirming the positive influence conferred by the use of BAP as a cytokinin on leaf organogenesis from peach. However, other studies have reported better results on peach leaf regeneration when cytokinins other than BAP were added to the culture medium. As observed by Zhou et al., (2010), TDZ as cytokinin (9.08 µM TDZ + 0.54 µM IBA) induced the best regeneration of shoots (71.7%, with mean number of shoots of 5.74 ± 3.24) from leaves of the peach rootstock Nemaguard. Additionally, San et al., (2015) observed a positive effect conferred by the use of TDZ instead of BAP as a cytokinin for shoot regeneration from Guardian® *in vitro* leaves. However, in this study, the presence of TDZ combined with NAA was not sufficient to induce shoot organogenesis, but the addition of the ethylene inhibitor STS at a concentration of 10 µM was an essential factor to obtain an organogenic response (regeneration rate of 16% with a mean number of shoots of 1.6). Our results showed that the use of TDZ induced a significant reduction in the regeneration of shoots from Hansen 536 leaves compared to WPM 11 medium, especially when TDZ was used in combination with NAA in the regeneration media. Similarly, in our study, the regeneration rates obtained by the use of KIN as a cytokinin were significantly lower (13.3% of regeneration) compared to the use of BAP alone; however, to our knowledge, this represents the first successful attempt to induce adventitious regeneration from peach leaves through

the use of KIN. Indeed, other authors reported the effect of KIN on caulogenesis from peach leaves, which did not lead to the regeneration of any adventitious shoot (Declerck and Korban, 1996; Escalettes and Dosba, 1993). Although all the studies mentioned above underline the difficulty in obtaining a versatile protocol to be applied for leaf organogenesis in different peach genotypes, it seems that the presence of the petiole as part of the starting leaf explant represents a critical factor for a successful adventitious shoot regeneration from leaves of peach rootstocks and cultivars, as also reported by Gentile et al. (2002) and by other authors in different *Prunus* species (Escalettes and Dosba, 1993; Liu and Pijut, 2008; Miguel et al., 1996). Indeed, in all these studies, including ours, most of the regenerated adventitious shoots were observed at the petiole. Several studies carried out on *Prunus* spp. have also demonstrated that the interaction among different factors, other than the medium composition, concur in the organogenic response of *in vitro* leaf explants, such as plant source conditions, the age of explant, cut side and leaf orientation on the culture substrate (Pérez-Tornero et al., 2000; Zhou et al., 2010). In our study, we observed a very low frequency of leaf organogenesis when Hansen 536 explants were cultured with the adaxial surface in contact with WPM11 medium (Supplementary Figure S2; data not shown) in contrast with the results obtained by Zong et al., (2019) on the same genotype and by Gentile et al., (2002) on peach cultivars. Several authors have examined the influence of leaf orientation on explant regeneration frequency in different plant species, however without obtaining a common response (Bhatia et al., 2005; Cardoso and Habermann, 2014; James et al., 1988; Mazumdar et al., 2010; Meng et al., 2004; Pérez-Tornero et al., 2000). In general, it seems that a higher regeneration efficiency should be achieved when the wounded surface of the leaf is in contact with the medium, probably due to a better absorption of nutrients and PGRs from the substrate (Pérez-Tornero et al., 2000; Sarwar and Skirvin, 1997). Furthermore, the different organogenic responses of the leaf in its two different orientations can be connected to the total surface area that is in contact with the substrate. Positioning the abaxial side of the explant on the medium avoids the typical curling of both ends of the leaf that occurs when it is oriented with the adaxial surface on the substrate (Bhatia et al., 2005; Mazumdar et al., 2010).

In the present study, we also investigated the influence of different concentrations of STS supplemented in the culture medium on the regeneration frequency and mean number of shoots from Hansen 536 leaves. Different authors stated that STS has a positive role in shoot organogenesis from leaf tissues of *Prunus* spp., including peach (Burgos and Alburquerque, 2003; Petri and Scorza, 2010; San et al., 2015). This effect seems to be associated to the action of Ag+ ions present in this compound, which block ethylene signal transduction induced by *in vitro* cultured explants (Burgos and Alburquerque, 2003). The ability of Ag+ ions to inhibit ethylene action seems to prevent some of its negative effect during shoots organogenesis (Chi et al., 1990; Kumar et al., 2009). In our study, we observed a significant increase in the average number of shoots per explant when STS was used at a concentration of 10 µM. Furthermore, the effect of this ethylene inhibitor on Hansen 536 leaf organogenesis was dose-dependent; in particular, when STS was used at concentrations higher than 10 µM, adventitious shoot regeneration was progressively inhibited. Our results together with previously published data reported by San and collaborators (2015) suggest 10 µM as the optimal concentration of STS capable of improving the adventitious regeneration of peach leaves. Other studies have already reported that STS and similar compounds, like silver nitrate, are biologically active at very low concentrations for some plant species (Chi et al., 1990; Möllers et al., 1992); in these cases, if the maximum effective concentration of STS is reached, toxic effects on plant tissues are induced and regeneration responses are inhibited, as also observed in our study. This also suggests that, at the right level, the presence of ethylene in the tissue culture is still needed for an efficient organogenic response, as also reported by other studies (Dimasi-Theriou and Economou, 1995; Trujillo-Moya and Gisbert, 2012). Thus, as remarked by Petri and Scorza (2010), the optimum STS concentration in *Prunus* spp. and other plant species is often

genotype-dependent, making essential the evaluation of STS impact on shoot regeneration from each cultivar or rootstock.

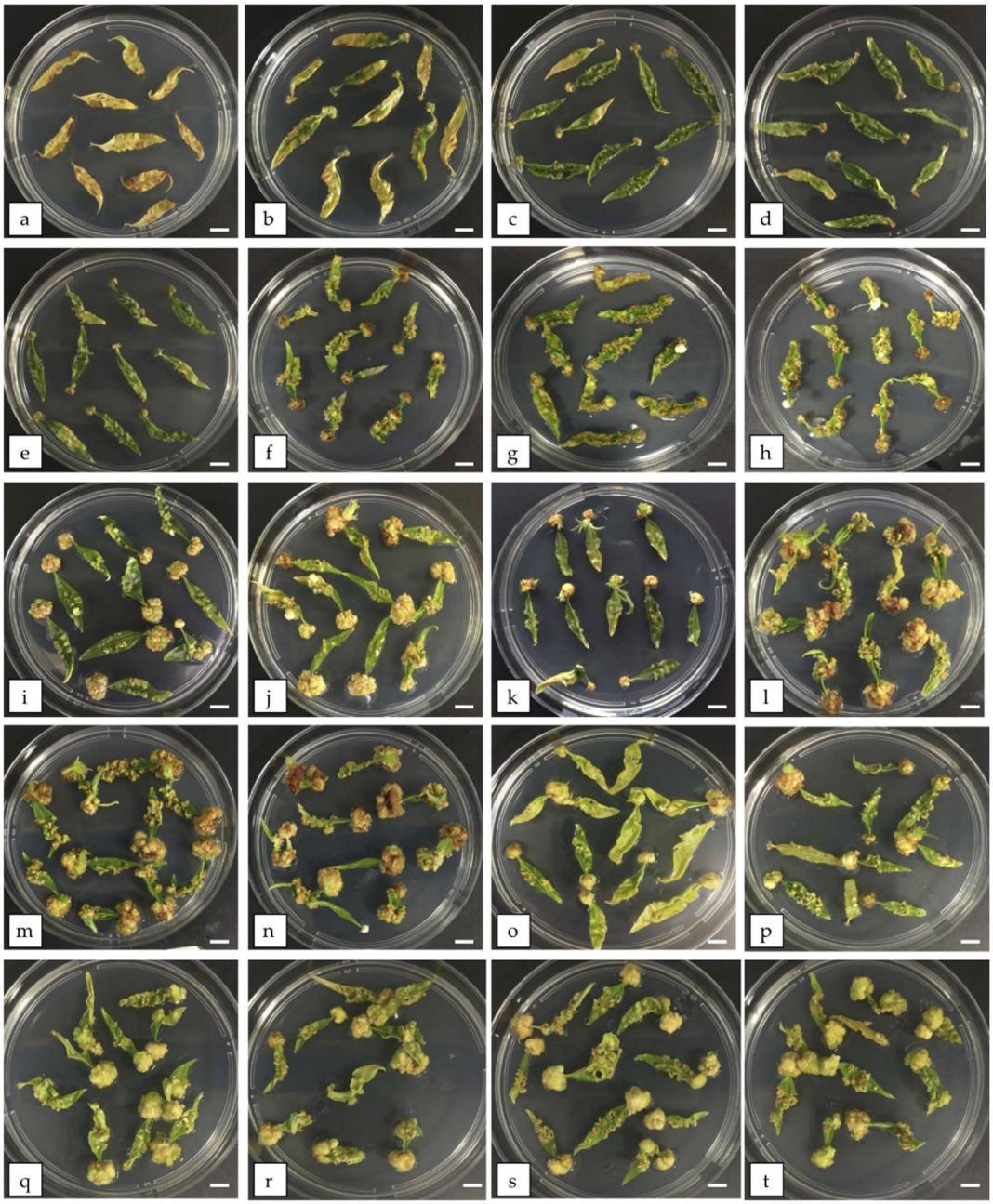
Lastly, the influence of different antibiotics included in the WPM 11 medium on the regeneration efficiency from Hansen 536 leaves was evaluated; this trial aimed to test only the antibiotics commonly used during *Agrobacterium*-mediated transformation trials to avoid bacterial persistence in the medium post-transformation. Several studies reported the positive effect of antibiotics on organogenic processes in different plant species included *Prunus* spp. (Borrelli et al., 1992; Bosela, 2009; Burgos and Alburquerque, 2003; Dai and Castillo, 2007; Hammerschlag et al., 1997; Holford and Newbury, 1992; Nakano and Mii, 1993; Nauerby et al., 1997; Orlikowska et al., 1995; Shehata et al., 2010; Silvestri et al., 2016; Yepes and Aldwinekle, 1994; Yu et al., 2001; Yu and Wei, 2008). We observed that carbenicillin (238 µM) combined with cefotaxime (210 µM) improved the average number of shoots per explant compared to the control, whereas the same antibiotics used alone at higher concentrations decreased regeneration efficiency (Table 3). Carbenicillin has been often tested to improve plant organogenesis, and it seems that the positive effect reported by different studies can be related to the byproducts released during its breakdown, which show an auxin-like activity with beneficial effects on shoot development in various plant species (Burgos and Alburquerque, 2003; Hammerschlag et al., 1997; Holford and Newbury, 1992; Nakano and Mii, 1993; Orlikowska et al., 1995; Shehata et al., 2010; Yu et al., 2001). In our study, a concentration of carbenicillin higher than 238 µM has probably induced an excess in auxin activity in peach leaf tissues, which led to a considerable reduction of regeneration frequency from Hansen 536 leaves. Similarly, a toxic effect of this antibiotic used at concentrations higher than 238 µM was also observed by other authors working with different plant species other than *Prunus* spp. (Grzebelus and Skop, 2014; Nauerby et al., 1997; Sarma et al., 1995). Differently from carbenicillin, the chemical conformation of cefotaxime does not suggest the production of auxin-like compounds as byproducts during its breakdown (Holford and Newbury, 1992; Sujana and Naidu, 2011); thus, additional studies are needed to understand the mode of action of this antibiotic and its involvement in plant metabolism. Our results show that the use of cefotaxime at concentrations higher than 210 µM leads to a reduction in regeneration efficiency; the negative effect of cefotaxime on plant organogenesis has also been reported in other studies (Holford and Newbury, 1992; Sarma et al., 1995; Tambarussi et al., 2015), even though the neutral or beneficial impacts of this antibiotic on morphogenesis of several plant species (Borrelli et al., 1992; Bosela, 2009; Dai and Castillo, 2007; Nakano and Mii, 1993; Nauerby et al., 1997; Rugini and Caricato, 1995; Yepes and Aldwinekle, 1994; Yu et al., 2001; Yu and Wei, 2008), including *Prunus* spp. (Burgos and Alburquerque, 2003), have been the main outcome observed until date.

In conclusion, we set up a valid and reproducible adventitious shoot regeneration system from Hansen 536 leaf tissues with a frequency up to 53% when explants were cultured on WPM medium enriched with 15.5 µM BAP. The established protocol has improved the regeneration efficiency of Hansen 536 leaves compared with maximum results reported by Zong et al. (2019) (regeneration frequency up to 36%) on the same genotype, probably due to the interaction of different factors, such as different sources of starting plant material, medium composition, leaf orientation on the substrate and type of leaf incision. Furthermore, we identified the optimal concentration of STS which significantly improved shoot regeneration by increasing two-fold the average number of shoots per explant. In addition, the regeneration medium enriched with carbenicillin combined with cefotaxime at the concentration tested induced a significant increase of regenerating shoots per leaf. This result has particular relevance for the genetic transformation of this peach genotype. In fact, these kinds of antibiotics are normally used during genetic transformation processes to contain *Agrobacterium* persistence in the medium after transforming plant tissues; therefore, it is essential to avoid negative effects on regeneration efficiency that could be caused by these compounds (Burgos and Alburquerque, 2003).

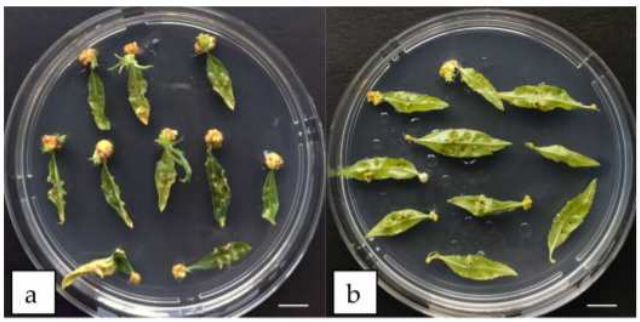
The overall results obtained in this study should improve the knowledge on factors controlling peach leaf organogenesis, and the results achieved by the best combination of the tested factors look promising for the optimization of new genetic transformation protocols in Hansen 536 and other peach rootstocks and cultivars.

[1]This chapter has been published: **Ricci, A.**, Capriotti, L., Mezzetti, B., Navacchi, O., Sabbadini, S. (2020). Adventitious shoot regeneration from *in vitro* leaf explants of the peach rootstock Hansen 536. *Plants*, *9*(6), 755. DOI: 10.3390/plants9060755

5. Supplementary material



Supplementary figure 1. Caulogenesis and leaf regeneration from Hansen 536 explants placed on WPM 1 - WPM 20 (a - t) media after five weeks from the beginning of the experiment (bar = 1 cm).



Supplementary figure 2. Hansen 536 leaf explants after five weeks of culture with the abaxial side **(a)** or the adaxial side **(b)** on WPM11 medium (bar = 1 cm).

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CHAPTER 5

RESEARCH SECTION

APPROACHES APPLIED TO INDUCE REGENERATION, THROUGH ORGANOGENESIS AND SOMATIC EMBRYOGENESIS, IN THE PEACH ROOTSTOCK GF677 (*P. persica x P. amygdalus*) AND IN SEVERAL PEACH CULTIVARS USING LEAVES, ANTHERS AND MATURE SEEDS AS STARTING EXPLANTS

1. Introduction

A key step in an *Agrobacterium*-mediated gene transfer system is the optimization of suitable procedures for adventitious shoots regeneration. The understanding of key growth mechanisms that underlie the regeneration processes would be useful in elucidating optimal *in vitro* cultures conditions for genetic transformation in the *Prunus* genus, including *P. persica* L. (Burgos et al., 2007).

Since the end of '80s, many achievements and failures in terms of peach regeneration via organogenesis and somatic embryogenesis (SE), respectively have been reported, both using adult and juvenile tissues, including several examples where really good regeneration rates were recorded (extensively reviewed in chapter 2 of this thesis). Few years ago, Sabbadini et al. (2015) established an *A. tumefaciens*-mediated transformation protocol using meristematic bulk slices of the peach hybrid rootstock GF677 as starting material; after eight months of selection with kanamycin, the transgenic status of two independent lines was confirmed. A similar protocol has been applied by the same authors to the peach cultivar Big Top, showing its inefficiency in obtaining transgenic shoots (unpublished data).

Consequentely, the main goal of the work described in this chapter was to establish alternative efficient protocols to achieve adventitious shoot regeneration from other somatic tissues and from juvenile tissues of *P. persica* L. (including GF677 and Big Top), more appropriate to obtain transgenic peach lines in future *Agrobacterium*-mediated transformation experiments. For this purpose, we adapted strategies already used successfully in peach by our group as well as in other *Prunus* spp. (Canli and Tian, 2008, 2009; Carmona-Martin and Petri, 2020; Urtubia et al., 2008; Wang et al., 2011, 2013; Petri et al., 2008). As demonstrated by Sabbadini et al. (2019) in another fruit tree species, the success of *in vitro* plant regeneration and the competence of genetic transformation greatly depends on the genotype of the species of interest; thus a large number of peach genotypes has been considered for this study.

2. Materials and methods

2.1. Organogenesis from in vitro leaf explants of the peach rootstock GF677 (P. persica x P. amygdalus) and the peach cultivar Big Top

2.1.1. Establishment of in vitro shoots

Shoot tips (0.5 cm long) were cut from shoots (approximately 10 cm long) of 10-year-old greenhouse-grown GF677 *(P. persica x P. amygdalus)* and Big Top trees at Vitroplant Italia, Cesena, Italy and used to establish *in vitro* proliferating cultures. The shoot tips were promptly surface sterilized by washing them in 1% (V/V) sodium hypochlorite solution for 15 min, followed by three washes with sterile distilled water for 5 min in a laminar-flow hood. Explants were then placed on multiplication medium composed of McCown Woody Plant Medium (WPM) (Duchefa Biochemie, Haarlem, The Netherlands) basal salts and vitamins (Lloyd and McCown, 1980), 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000 (B&V, Reggio Emilia, Italy) supplemented with N^6-benzylaminopurine (BAP) (4.4 or 5.5 μM) (Duchefa Biochemie, Haarlem, The Netherlands) and α-naphtalene acetic acid (NAA) (0.05 or 0.1 μM) (Duchefa

Biochemie, Haarlem, The Netherlands) for GF677 and Big Top, respectively. The final pH value was adjusted to 5.7-5.8 with KOH prior adding gelling agent and autoclaving at 121°C for 20 min. *In vitro* cultures were incubated in a growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol/m^2/s) provided by white fluorescent tubes, and they were periodically subcultured (2-week intervals) on fresh multiplication medium for a total of three subcultures. Proliferating shoot cultures were then transferred to the elongation medium in order to provide expanded leaves for this study. Preparation and composition of the elongation medium were the same as for the multiplication medium, except for plant growth regulators (PGRs) concentrations, corresponding to 0.45 µM BAP and 0.1 µM NAA and 0.55 µM BAP and 0.2 µM NAA for GF677 and Big Top, respectively. Elongation shoot cultures were kept in a growth chamber at 24 ± 1°C under a photoperiod of 16-h light (70 µmol/m^2/s) provided by white fluorescent tubes for three weeks and then use for shoot regeneration tests.

2.1.2. Effect of different combinations and concentrations of plant growth regulators (PGRs)

Apical expanding leaves from 3-week old elongated shoot cultures of the peach rootstock GF677 and the peach cultivar Big Top were used as starting explants in this study. In order to determine PGRs combinations capable of inducing shoot organogenesis from GF677 and Big Top leaf explants, the same approach already used with the peach hybrid rootstock Hansen 536 has been applied. Briefly, wounded abaxial surfaces of apical expanding leaves from 3-week-old elongated shoot cultures were placed in contact with regeneration media enriched with cytokinins, as kinetin (KIN) (14, 16.3 or 19 µM), BAP (11, 13.3 or 15.5 µM) or thidiazuron (TDZ) (2.25, 4.5 or 9 µM) and with auxin, as NAA (0.25 µM), for a total of twenty different media combinations (all PGRs produced by Duchefa Biochemie, Haarlem, The Netherlands). Leaves were kept in darkness at 24 ± 1°C for three weeks and then moved to fresh media and exposed to light (the same conditions described above) at 24 ± 1°C for two weeks. Furthermore, a second trial aimed to deeply investigate the impact of TDZ on shoot organogenesis from GF677 leaf explants by placing them on regeneration media supplemented with lower concentrations of TDZ (2.25, 1.13, 0.56 or 0.28 µM) used alone or in combination with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (0.1 µM) (Duchefa Biochemie, Haarlem, The Netherlands). The general approach for adventitious shoot regeneration was the same as the first experiment described above. Lastly, the influence of other PGRs added to the basal regeneration medium, alone or combined (Table 1), on the regeneration efficiency of Big Top leaves was evaluated using the same regeneration protocol previously described (some of these PGRs were used for peach regeneration for the first time in this study). Filter-sterilised zeatin (ZEA), its metabolic precursor isopentenyl adenine (2ip) and gibberellic acid (GA_3) (Duchefa Biochemie, Haarlem, The Netherlands) were added to the regeneration medium after autoclaving and cooling down to 50°C.

Table 1. Different combinations of PGRs used in regeneration media for Big Top

Plant growth regulators (PGRs) (µM)					
ZEA	**2ip**	**BAP**	**NAA**	**TDZ**	**GA_3**
2.5	-	8.9	-	-	-
2.5	-	8.9	0.12	-	-
-	2.5	8.9	-	-	-
-	2.5	8.9	0.12	-	-
-	-	-	-	2.25	0.3
-	-	-	-	2.25	3
-	-	-	-	2.25	7.5

For each genotype-treatment combination in all regeneration experiments, a total of fifty explants were prepared (ten leaves per Petri dish) and three independent trials were performed. Data on regeneration efficiency, expressed as the percentage of leaves regenerating at least one shoot per total leaves treated were recoreded after five

weeks from the beginning of the experiment. The results acquired were analyzed by one-way ANOVA using Statistica 7 software (Statsoft Tulsa, CA, USA), and means were separated using the Newman–Keuls test ($p < 0.05$).

2.2. SE from in vitro leaf explants of the peach rootstock GF677 and the peach cultivar Big Top

2.2.1. Plant material and induction of SE

Improving protocols already assessed for the peach rootstock Hansen 536 (described in chapter 2), leaves from 3-week old elongated shoot cultures of peach rootstock GF677 (*P. persica x P. amygdalus*) and cultivar Big Top, obtained following protocols described above, were used as starting material in this study for induction of SE. Leaves choice and explants preparation were carried out following the protocol reported in the previous chapter. Briefly, the wounded abaxial surface of each apical expanding leaf was cultured in contact with two SE induction media, supplemented with 4.5 or 9 µM 2, 4-D. The basal medium consisted of WPM basal salts and vitamins (Lloyd and McCown, 1980), 20 g L^{-1} sucrose, and 7 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, The Netherlands). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min, and then 25 ml of medium were poured into sterile plastic Petri dishes (9 cm x 1.5 cm). Leaves were cultured in darkness at 24 ± 1°C for seven weeks and then exposed to light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for additional five weeks. When cream-colored calli appeared, they were transferred to PGRs-free medium for the potential development of proembryonic masses (PEM) and eventually somatic embryos. Preparation and basal composition of the PGRs-free medium were the same as for SE induction medium. Cultures are maintained under light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for six weeks. For each genotype-treatment combination of this preliminary SE trial, five Petri dishes with a total of fifty explants were prepared (ten leaves per dish). The experiment was repeated twice.

2.3. SE from anthers of peach cultivars Big Top, Tardibelle and Big Bang

2.3.1. Plant material and induction of SE

Anthers from unopened flowers of one-year old dormant cuttings of peach cultivars Big Top, Tardibelle and Big Bang (provided by Acciarri, Fermo, Italy) were used as starting explants in this study for stimulation of SE (Figure 1 a, b, c). The unopened flowers were surface sterilized for 10 min with 1% (V/V) sodium hypochloride solution containing a few drops of Tween-20 per 100 ml solution, rinsed three times with sterile distilled water and placed in sterile plastic Petri dishes. Anthers were carefully removed from sterile unopened flowers under a stereomicroscope and horizontally placed on SE induction media. The SE induction media consisted of WPM basal salts and vitamins (Lloyd and McCown, 1980), 20 g L^{-1} sucrose, and 7 g L^{-1} plant agar, supplemented with 4.4 µM BAP and 2, 4-D (4.5 or 9 µM) (all reagents produced by Duchefa Biochemie, Haarlem, The Netherlands) (Table 2). The pH was adjusted to 5.7-5.8 before autoclaving at 121°C for 20 min, and then 25 ml of medium were poured into sterile plastic Petri dishes (9 cm x 1.5 cm). Anthers were incubated in darkness at 24 ± 1°C for seven weeks and then exposed to light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for additional five weeks. When cream-colored calli appeared, they were transferred to PGRs-free medium for the potential development of PEM and eventually somatic embryos. Preparation and basal composition of the PGRs-free medium were the same as for SE induction medium, except for the addition of 0.5 g L^{-1} activated charcoal (Duchefa Biochemie, Haarlem, The Netherlands). Cultures are maintained under light (16-h photoperiod at a light intensity of 70 µE $m^{-2}s^{-1}$) at 24 ± 1°C for six weeks.

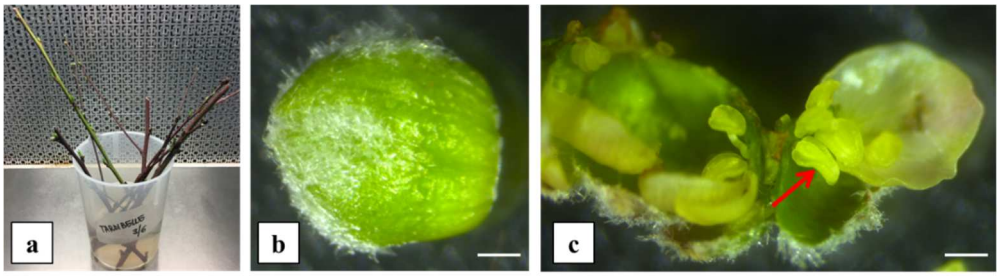

Figure 1. Plant material used as starting explants in this study: **(a)** One-year old dormant cuttings and **(b)** unopened flower of peach cultivar used as source of starting explants in the SE induction experiment (bar = 1 mm). **(c)** Sterile opened flower of peach cultivar. The arrow indicates the anther ready to be placed in the SE induction medium (bar = 1 mm).

2.4. Organogenesis from hypocotyl slices and cotyledons of peach cultivars Royal Summer, Red Pearl and Rome Star

2.4.1. Plant material, explant preparation and general regeneration strategy

Mature seed hypocotyl slices and cotyledons from the peach cultivars Royal Summer, Red Pearl and Rome Star were used as starting explants in this study. Seeds sterilization and explants preparation were carried out following protocols reported by Gonzalez-Padilla et al. (2003). Following the 2019 season harvesting of mature peaches (managed by Vitroplant Italia, Cesena, Italy), the mesocarp (flesh) was removed from the endocarp (stone), which, with the enclosed seed, was cleaned using 1% (V/V) sodium hypochlorite solution for a couple of hours and allowed to air dry 3-4 days at room temperature. Seeds were kept at 4°C until use for shoot regeneration trials. After the endocarp had been removed using a nutcracker, the seeds were sterilized by washing them in 1% (V/V) sodium hypochlorite solution, containing few drops of Tween-20 per 100 ml solution, for 20 min, followed by three washes with sterile distilled water in a laminar-flow hood. Disinfected seeds were soaked in sterile water overnight at 4°C and the seed coats removed with a scalpel. For each embryonic axis, the epicotyl (E) and the radicle (R) were discarded, and the hypocotyl was cut into two cross-sections (0.5-1 mm), and then placed in the regeneration medium (Figure 2 a, b). Cotyledon explants were prepared following the procedure reported by Wang et al. (2013). In particular, they were wounded three times in the adaxial side where the embryonic axis was, considering that this area has high morphogenic ability in other species (Canli and Tian, 2009). The wounded areas of cotyledon explants were then placed in contact with the regeneration medium (Figure 2 c, d).

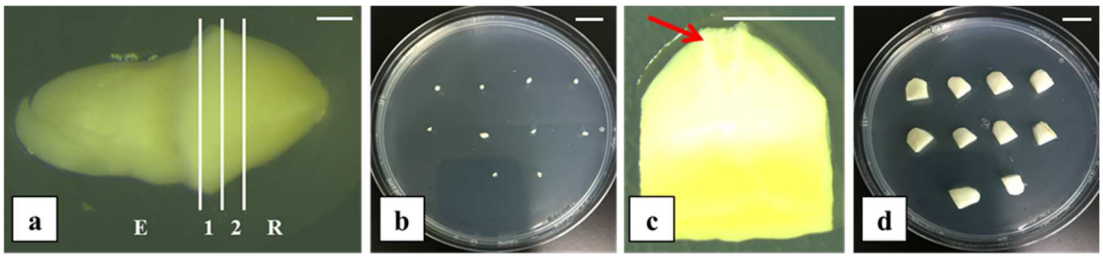

Figure 2. Explants from mature seeds of *P. persica* L. cultivars used in this study: **(a)** Embryonic axis, where 1 and 2 represent the hypocotyl slices used as starting explants in the regeneration experiments (bar = 1 mm). The epicotyl (E) and radicle (R) were not cultured in the regeneration medium (bar = 1 cm) **(b)**. Peach cotyledon **(c)** ready to be placed in the regeneration medium **(d)** (bar = 1 cm). The arrow indicates the area where the embryonic axis was and regeneration occurs.

The starting explants were cultured on Quoirin and Lepoivre (QL) basal salts and vitamins (Duchefa Biochemie, Haarlem, The Netherlands) (Quoirin and Lepoivre, 1977), 20 g L^{-1} sucrose, and 7 g L^{-1} plant agar, supplemented with 7.5 µM TDZ and 0.25 µM 3-indolebutyric acid (IBA) (Duchefa Biochemie, Haarlem, The Netherlands). The pH was adjusted to 5.7 before autoclaving at 121°C for 20 min, and then 25 ml of regeneration

medium were poured into sterile plastic Petri dishes (9 cm x 1.5 cm). After explants were placed on regeneration medium, they were incubated in darkness at 23 ± 1°C for two weeks and then exposed to light with a 16-h photoperiod (20-25 µmol m^{-2} s^{-1}, cool-white fluorescent lamp) at 23 ± 1°C for six weeks. The explants were not transferred to fresh medium during whole regeneration experiments.

2.4.2. Study of factors affecting adventitious shoot regeneration

In order to investigate the impact of 2, 4-D, a protocol (with modifications) optimized for *P. domestica* L. has been tested (Petri et al., 2008). Thus, both types of explants (hypocotyls slices and cotyledons) were placed on the regeneration medium supplemented with 9.05 µM 2, 4-D for two days in darkness at 23 ± 1°C and then moved to regeneration medium, or explants were cultured directly onto regeneration medium without 2, 4-D. In a second experiment, the addition of silver thiosulphate (STS) to the regeneration medium was evaluated by placing both types of starting explants on regeneration medium supplemented with 30 µM STS prior to autoclaving. The STS stock solution was prepared as described in the previous chapter. Explants were placed on regeneration medium supplemented with 30 µM STS during the whole experiment, or explants were cultured directly onto regeneration medium without STS. Regeneration frequency expressed as the percentage of explants regenerating at least one shoot per total explants treated was recorded after eight weeks from the beginning of regeneration trials. The results acquired were analyzed by one-way ANOVA using Statistica 7 software (Statsoft Tulsa, CA, USA), and means were separated using the Newman–Keuls test ($p < 0.05$).

3. Results

3.1. Organogenesis from in vitro leaf explants of the peach rootstock GF677 and the peach cultivar Big Top

In order to determine PGRs combinations capable of inducing shoot organogenesis from GF677 and Big Top leaf explants, the same strategy already used with the peach hybrid rootstock Hansen 536 has been attempted. Two-three weeks after incubation in darkness, calli developed from petioles as well as around the wounds made on leaf surface of both genotypes (Figure 3 a, b). After five weeks of culture, about 100% caulogenesis was observed on both GF677 and Big Top leaf explants placed on most of the used regeneration media (except for the medium without PGRs, where no calli formation was observed) (data not shown). Generally, there was no successful shoot organogenesis induction from both GF677 and Big Top leaf explants on any of the regeneration media used, even thought extremely low rates of regeneration efficiency were recorded when GF677 leaf explants were placed on basal medium supplemented with 2.25 µM TDZ used alone or in combination with 0.25 µM NAA (2% and 4%, respectively) (Figure 3 c, d).

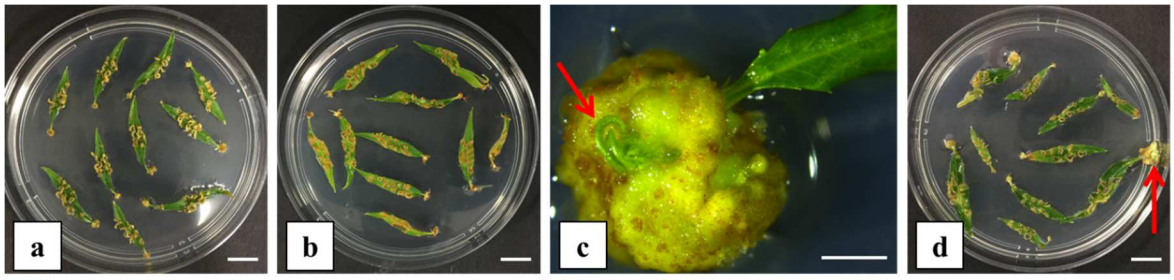

Figure 3. Organogenesis experiment on peach leaves: Calli formation from **(a)** GF677 and **(b)** Big Top cultured on basal regeneration medium supplemented with 13.3 µM BAP and 0.25 µM NAA in dark conditions after 2-3 weeks from the beginning of the trial (bar = 1 cm). Adventitious shoot regenerating from petiole of GF677 leaf after three **(c)** and five **(d)** weeks of culture on basal regeneration medium supplemented with 2.25 µM TDZ (bar = 1 mm figures c, and 1 cm figure d). Arrows indicates regenerating adventitious shoots.

Even though successfull shoot organogenesis has not been obtained in these experiments, useful data on morphogenesis from both GF677 and Big Top leaves were recorded and used for further organogenesis induction studies. One possible step forward could be the use of different concentrations of TDZ as well the use of a different type of auxin in the regeneration medium, more suitable maybe to obtain adventitious shoots from GF677 leaves in future experiments. Despite of similar caulogenesis rates as well as regeneration rates between GF677 leaves cultured in the presence of lower concentrations of TDZ used alone or in combination with 0.1 µM 2, 4-D, and those used before, the results recorded after five weeks from the beginning of the second trial showed a slightly better value of regeneration frequency (up to 12%) when 2.25 µM TDZ was used in combination with 0.1 µM 2, 4-D (Figure 4 a, b). In the third experiment, there was no successful adventitious regeneration from Big Top leaf explants on any of the media tested, even thought caulogenesis (approximately 100% most of time) were obtained when the explants were culture in regeneration media containing ZEA, 2ip or BAP applied alone or in combination with NAA (data not shown). Furthermore, there was no successful morphogenesis (caulogenesis as well as adventitious shoots regeneration) from Big Top leaf explants on regeneration media enriched with TDZ in combination with GA_3. In particular, concentrations of GA_3 equal or higher than 0.3 µM increasead necrosis progressively; in particular, when GA_3 was used at 0.3, 3 or 7.5 µM, the rate of necrosis expressed as the percentage of necrotic leaves per total leaves treated increasead drastically to 28%, 57% and 100%, respectively after 5 weeks of culture (Figure 4 c, d, e).

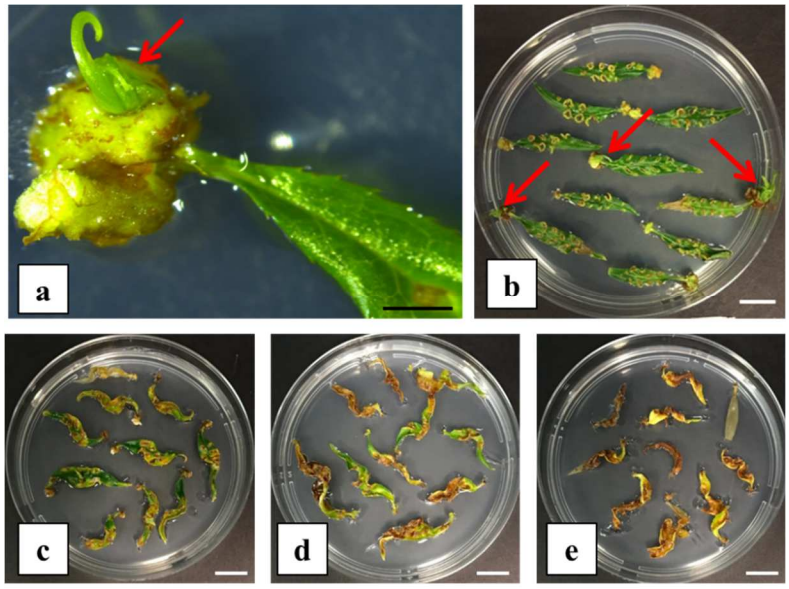

Figure 4. Organogenesis experiments on peach leaves: Adventitious shoot regenerating from petiole of GF677 leaf after three **(a)** and five **(b)** weeks of culture on basal regeneration medium supplemented with 2.25 µM TDZ 0.1 µM 2, 4-D (bars = 1 mm and 1 cm, respectively). Arrows indicate regenerating adventitious shoots. Leaves explants of Big Top cultured on basal regeneration medium supplemented with 2.25 µM TDZ in combination with **(c)** 0.3, **(d)** 3 or **(e)** 7.5 µM GA_3 after five weeks from the beginning of the experiment (bar = 1 cm).

3.2. SE from in vitro leaf explants of the peach rootstock GF677 and the peach cultivar Big Top

In this study, there was no successful SE stimulation from both GF677 and Big Top on any of the media used, even thought caulogenesis was obtained. There were no significant differences for callus type and size as well as caulogenesis rate between rootstock and cultivar leaf explants cultured on both SE induction media. Basically after 12 weeks of culture, approximately 100% caulogenesis was obtained expressed as percentage of leaves with callus per total number of treated leaves. Cream-colored calli grew on petioles and developed around the wounds made on abaxial side, covering entirely the surface of the leaves (Figure 5 a, b, c, d). After caulogenesis data were recorded (12 weeks after

the beginning of the trial), whole explants (approximately 10 per cultivar-treatment, randomly chosen) were gently moved to the PGRs-free medium, where they all started to become brownish 2-3 weeks after transferring (Figure 5 e).

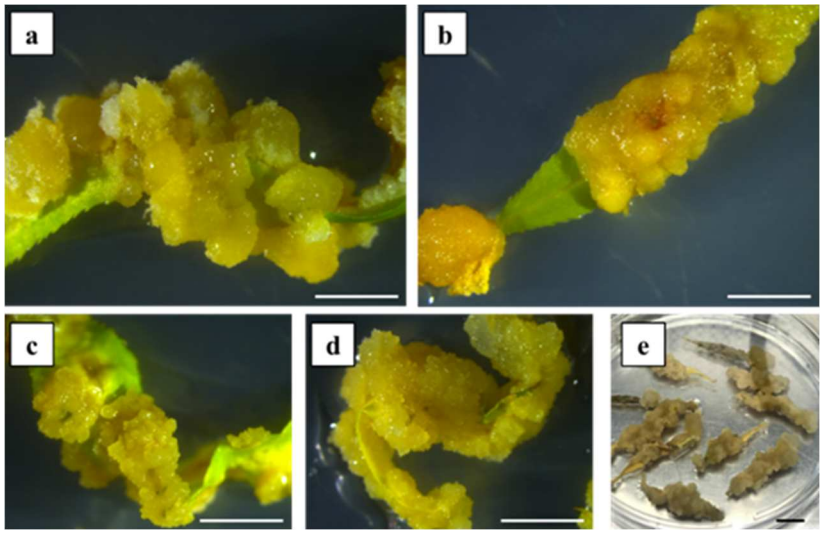

Figure 5. SE experiment on peach leaves: **(a, b)** Cream-colored calli developed from GF677 cultured on 4.5 µM 2, 4-D and 9 µM 2, 4-D, respectively. **(c, d)** Cream-colored calli developed from Big Top cultured on 4.5 µM 2, 4-D and 9 µM 2, 4-D, respectively. The images were taken after 12 weeks from the beginning of the experiment. **(e)** Type of brownish calli from peach leaves cultured on PGRs-free medium after 14-15 weeks from the beginning of the experiment (bar = 1 cm).

3.3. SE from anthers of peach cultivars Big Top, Tardibelle and Big Bang

In this experiment, there was no successful SE induction from the three peach cultivars on any of the media tested, even thought caulogenesis was observed. When Big Top, Tardibelle and Big Bang anthers were cultured on SE induction medium enriched with BAP 4.4 µM 2, 4-D 4.5 µM, explants produced cream-colored calli after 12 weeks from the beginning of the culture (Figure 6 a, b, c). In particular, caulogenesis frequencies were 78.2%, 65.7%, and 82.4% for Big Top, Tardibelle and Big Bang, respectively (Table 2). Furthermore, the same type of cream-colored calli was obtained from anthers when BAP 4.4 µM 2, 4-D 9 µM were supplemented in the SE induction medium; caulogenesis rates of 76.5%, 53.8% and 92% were recorded for Big Top, Tardibelle and Big Bang, respectively after 12 weeks from the beginning of the experiment (Table 2). After caulogenesis data were annotated (12 weeks after the beginning of the trial), cream-colored calli (approximately 10 per cultivar-treatment, randomly chosen) were gently moved to the PGRs-free medium but they all started to necrotize 4-5 weeks after transferring.

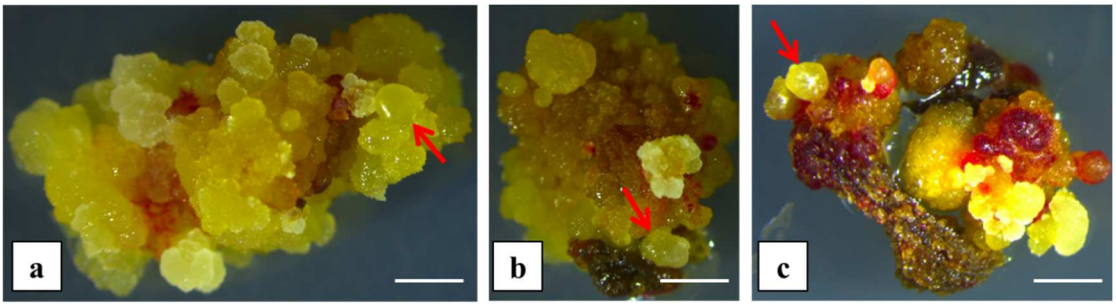

Figure 6. SE experiment on peach anthers: Cream-colored calli developed from **(a)** Big Top, **(b)** Tardibelle and **(c)** Big Bang anthers cultured on BAP 4.4 µM 2, 4-D 4.5 µM; the images were taken after 12 weeks from the beginning of the experiment. The arrows indicate the type of cream-colored calli transferred to PGRs-free medium (bar = 1 mm).

Table 2. Frequency of caulogenesis obtained from anthers of *P. persica* L. cultivars cultured in SE induction media

Cultivar	Frequency of caulogenesis (%) ± SE *	
	BAP 4.4 µM 2, 4-D 4.5 µM	BAP 4.4 µM 2, 4-D 9 µM
Big Top	78.2 ± 4.5	76.5 ± 5.9
Tardibelle	65.7 ± 8.1	53.8 ± 6.9
Big Bang	82.4 ± 4.9	92 ± 3.1

* Percentage of anthers developing callus per total number of treated anthers recorded after 12 weeks from the beginning of the trial. A total of 87, 35 and 74 anthers for Big Top, Tardibelle and Big Bang, respectively were placed on media supplemented with BAP 4.4 µM 2, 4-D 4.5 µM. A total of 51, 52 and 75 anthers for Big Top, Tardibelle and Big Bang, respectively were placed on media supplemented with BAP 4.4 µM 2, 4-D 9 µM. Statistical differences among cultivars were not calculated.

3.4. Organogenesis from hypocotyl slices and cotyledons of peach cultivars Royal Summer, Red Pearl and Rome Star

Adventitious shoot regeneration efficiency varied among the cultivars and the regeneration capacity seemed to be genotype-dependent. Both types of explants increased two-fold their initial size after the 2-weeks dark-incubation period (Figure 7 a, b), and adventitious shoots initially were observed within 3-4 weeks of culture from the beginning of regeneration trials. Adventitious regeneration in hypocotyl slices and cotyledons appeared mostly along the edge (Figure 7 c) and on the proximal surface (Figure 7 d), respectively. Hypocotyl sections and cotyledons were directly placed onto regeneration medium or they were initially placed onto regeneration medium supplemented with 9.05 µM 2, 4-D for two days and then moved to regeneration medium. The 2, 4-D treatment did not affect regeneration from hypocotyl sections on any of the cultivars tested (Figure 7 e). In cotyledons, no significant effect was recorded in relation to the use of 2, 4-D for both Royal Summer and Rome Star, but the frequency of regeneration decreased to 6.5% in the 2, 4-D treated cotyledons compared to the non-treated ones for Red Pearl (Figure 7 f). Furthermore, either types of explants were cultured on regeneration medium or they were cultured on regeneration medium supplemented with 30 µM STS during the whole regeneration experiment. The STS at the concentration applied did not affect the regeneration frequency in any of the cultivars tested (data not shown).

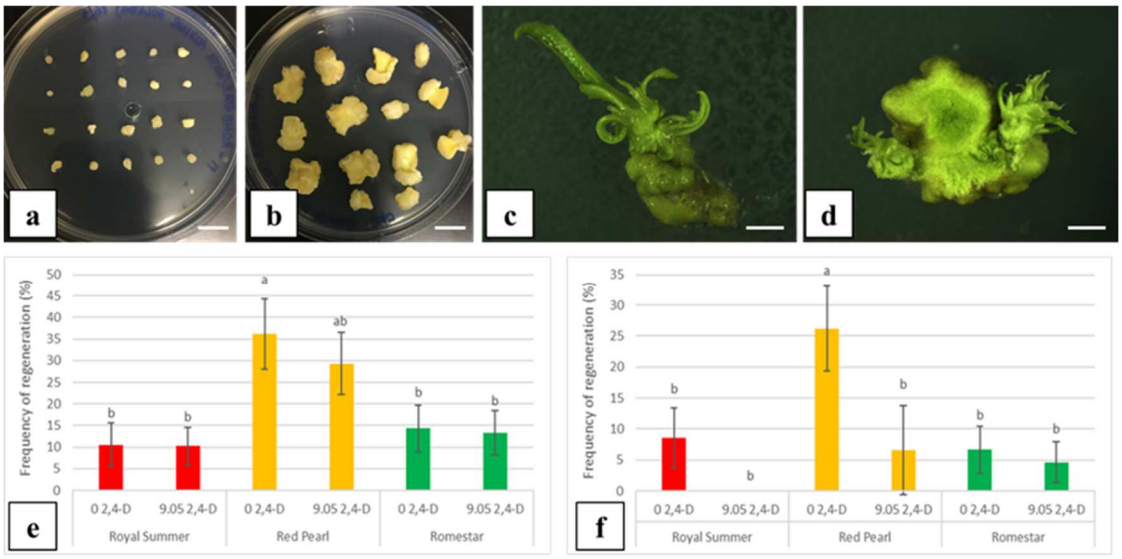

Figure 7. Organogenesis experiment on peach juvenile tissues: **(a)** Red Pearl hypocotyl slices and **(b)** cotyledons cultured on regeneration medium after 2 weeks in dark conditions (bar = 1 cm). Adventitious regeneration from **(c)** Red Pearl hypocotyl slice and **(d)** cotyledon. Pictures were taken after six weeks from the beginning of the trial (bar = 1 mm). Frequency of regeneration from **(e)** hypocotyl slices and **(f)** cotyledons expressed as (number of explants regenerating at least one shoot/total explants treated) x 100 recorded after 8 weeks from the beginning of the trial. The results were analysed by one-way ANOVA, and Newman-Keuls test ($p < 0.05$) ± SE (n = 60) was used to identify significant differences. Data reported in the picture represent the mean ± SE of three independent experiments.

4. Discussion

4.1. Organogenesis from in vitro leaf explants of the peach rootstock GF677 and the peach cultivar Big Top

It is widley known that adventitious shoot organogenesis largely depends on the type of hormonal stimulus applied to the plant tissue. In this study, none of the PGRs combinations tested induced a leaf organogenetic response in Big Top, while low regeneration rates (from 2% up to 12%) were recorded when leaf explants of GF677 were cultured on regeneration media enriched with TDZ used alone or in combination with NAA or 2, 4-D. Similarly, adventitious shoot regeneration from both Nemaguard and Guardian *in vitro* leaf explants has been induced using TDZ as cytokinin (9.08 and 6.81 μM, respectively) in the regeneration media (San et al., 2015; Zhou et al., 2010). In other studies (including ours shown in the previous chapter), adventitious shoot regeneration from different peach rootstocks and cultivars has been obtained mostly adding BAP as cytokinin to the regeneration media (Gentile et al., 2002; Zong et al., 2019). Furthermore, studies carried out on *Prunus* spp. have shown that organogenesis process depends also on starting plant material (genotype and age), cut side and explant orientation on the regeneration substrate (Carmona-Martin and Petri, 2020; Pérez-Tornero et al., 2000; Zhou et al., 2010). Thus, different developmental stages of the leaf as well as different wound patterns (by acting also on the mid vein for instance) might be evaluated in further leaf organogenesis studies using GF677 and Big Top as genotypes. In our preliminary study, the change of leaf orientation (from abaxial side to adaxial side) in contact with certain regeneration media tested above has not improved the leaf organogenetic response from GF677 and Big Top (data not shown). Despite the amount of time and efforts invested, an efficient adventitious shoot regeneration system via leaf organogenesis from these two important commercial genotypes has not been established. However, to the best of our knowledge, this represents the first successful attempt to induce adventitious regeneration from leaves of the peach rootstock GF677.

4.2. SE from the peach rootstock GF677 and several peach cultivars using in vitro leaf explants and anthers as starting explants

SE is defined as "the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes" (Williams and Maheswaran, 1986). As reviewed by Dodeman et al. (1997), the development of somatic embryos from somatic cells relies on the *in vitro* induction of SE pathway in differentiated cells, whereby PGRs and stress conditions are the two main artificial factors used to stimulate embryogenesis in somatic cells. In particular, at the beginning of 2000s, researchers observed that PGRs (especially auxins) play an essential role as inducers of the dedifferentiated somatic cells towards SE pathways (Fehér, 2005; Gaj, 2004; Jiménez and Thomas, 2005; Pasternak et al., 2002; Thomas and Jiménez, 2005). Two pathways, which are the direct and indirect SE have been investigated for SE induction. In one pathway, somatic embryos originate directly from somatic cells, and on the other somatic embryos originate from a dedifferentiated callus (Ji et al., 2011). To the best of our knowledge, SE has not been previously obtained in peach mature tissues but exclusively from peach immature embryos and cotyledon (as reviewed in chapter 2). This has been reported using induction media based on MS as basal salt mainly supplemented with 2, 4-D and BAP (Bhansali et al., 1990; Guohua and Yu, 2002; Scorza et al., 1990). 2, 4-D is considered the most efficient auxin for induction of SE pathway from immature zygotic embryos compared to other auxins such as NAA or β-naphthoxyacetic acid (NOA) (Druart, 1999). Although references cited as well as our previous study (showed in chapter 2) have been considered when designing these experiments, the results obtained from the trials showed that none of the media tested induced the development of somatic embryos from the somatic cells treated. In chapter 2 different attempts to induce SE in the peach hybrid rootstocks Hansen 536 and GF677 were described, using leaves from meristematic bulks and flowers respectively, as starting explants. Even though somatic

embryos have not been obtained in this work, cream-colored calli formation in both type of explants was observed and considered as promising starting point for further SE induction studies. One possible step forward can be the use of a different type of leaf (expanding leaf from elongated shoot culture instead of leaf from meristematic bulk), as well as anthers at different developmental stages as starting somatic tissues, more appropriate maybe to obtain somatic embryos in future SE experiments. Our knowledge on SE induction using anthers as starting explants is based on studies made on grapevine, where it is generally stated that embryos developed from *in vitro* cultured anthers arise from the diploid cells of the connective tissue and are therefore of somatic origin (Faure, 1996). The current investigation aimed to determine the proper balance of *in vitro* stimuli capable of inducing SE in a peach rootstock and cultivars by evaluating the impact of expanding leaves as well as younger anthers (that those described in chapter 2) cultured in different induction media consisted of WPM as basal salt supplemented with BAP and 2, 4-D. Several peach genotypes have been tested because the induction of SE in horticultural plants is genotype-dependent (Ji et al., 2011). Despite the use of different types of starting explant the situation at first sight has not improved, it would be worth continuing to work with them throught their culture on induction media enriched with many other concentrations and combinations of BAP as cytokinin and 2, 4-D as auxin. In fact, improving procedures already tested in our previous study and described in chapter 2, we were able to obtain cream-colored calli (which might be pre-embryogenic from our point of view) when anthers of Big Top, Tardibelle and Big Bang were cultured on SE induction media supplemented with BAP 4.4 µM in combination with 2, 4-D 4.5 or 9 µM. In conclusion the optimization of efficient regeneration protocol through SE in *Prunus persica* L. might become a useful tool for peach genetic improvement, as recovery of adventitious shoots through organogenesis of important peach genotypes (as GF677 and Big Top for instance) is challenging and time-consuming as we have broadly shown in this chapter.

4.3. Organogenesis from hypocotyl slices and cotyledons of peach cultivars Royal Summer, Red Pearl and Rome Star

Adapting strategies already used successfully with other *Prunus* spp., such as *P. armeniaca* L. (Wang et al., 2011, 2013), *P. avium* L. (Canli and Tian, 2008), *P. cerasifera* L. (Carmona-Martin and Petri, 2020), *P. domestica* L. (Petri et al., 2008), *P. insititia* L. (Carmona-Martin and Petri, 2020) and *P. salicina* L. (Canli and Tian, 2009; Urtubia et al., 2008), mature seed hypocotyl slices and cotyledons from *P. persica* L. cultivars Royal Summer, Red Pearl and Rome Star were used as starting explants in this study. Although additional replicates have to be performed to confirm the preliminary data shown here, the results obtained in this study are comparable to those reported for other peach genotypes (reviewed in chapter 2). Furthermore, shoots elongation, rooting and acclimatization protocols have to be still optimazed for the cultivars under evaluation. It is widely known that organogenesis process depends on the synergistic interaction between the endogenous hormonal content and suitable *in vitro* stimulus, together with tissue type and genotype involved (Carmona-Martin and Petri, 2020). Moreover, as stated by Petri and Scorza (2010), the effect of STS in *Prunus* spp. and other plant species is generally genotype-dependent, making crucial a meticulous STS impact assessment on regeneration from each genotype. Thus, different STS as well as 2, 4-D concentrations might be evaluated in further studies in order to optimize their use for each explant and genotype of interest. In this study, Red Pearl appeared as the best cultivar in terms of regeneration frequency showing up to 36% when hypocotyl sections were cultured on regeneration medium without 2, 4-D treatment. The overall preliminary data recorded in this study might improve the knowledge on conditions influencing peach organogenesis from juvenile tissues, and the results achieved look promising for the optimization of new regeneration protocols in Red Pearl and other peach cultivars and rootstocks, to enable insertion of a hairpin construct (ihp35s-PPV194) capable of inducing PPV resistance via PTGS.

In conclusion, in this chapter, we described different approaches (summarized in Figure 8) in order to induce

adventitious regeneration, through organogenesis and SE, in the peach hybrid rootstock GF677 and in several peach cultivars using different types of tissues. In particular, different regeneration protocols, via organogenesis, are available using leaves, hypocotyls slices and cotyledons of different peach genotypes, some of them showing a relatively high efficiency. Usually, with regeneration strategies that involve organogenesis as route of morphogenesis, the main problem remains the optimization of a regeneration protocol which basically is genotype-dependent, while the limitation of SE is the obtainment of somatic embryos, especially from peach somatic tissues. Protocols for adventitious regeneration are needed to optimize a suitable peach transformation system, in order to induce resistance to PPV in *Prunus persica* L. via PTGS.

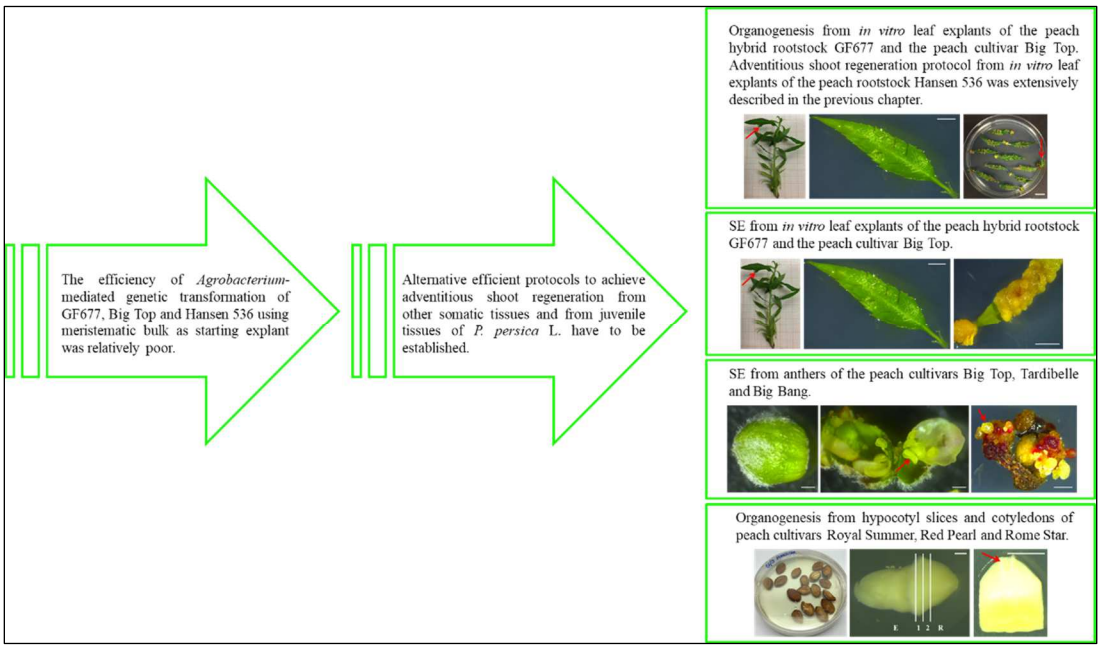

Figure 8. Strategies applied to induce regeneration, through organogenesis and SE, in the peach rootstock GF677 (*P. persica x P. amygdalus*) and in several peach cultivars using leaves, anthers and mature seeds as starting material.

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CHAPTER 6

RESEARCH SECTION

GENETIC TRANSFORMATION OF *IN VITRO* LEAF EXPLANTS OF THE PEACH ROOTSTOCK HANSEN 536 USING DIFFERENT SPECIES OF *AGROBACTERIUM* TO EXPRESS AN RNAi CONSTRUCT AGAINST PPV VIRUS

1. Introduction

Prunus persica L., one of the most commercially valuable species among *Prunus* spp., is especially susceptible to different pests and now is facing the emergency of the virus infection determined by the diffusion of the plum pox virus (PPV), also denominated as Sharka virus (Petri and Scorza, 2008).

A stimulating approach based on the exploitation of a natural mechanism for gene espression regulation, known as post-transcriptional gene silencing (PTGS), is being used to introduce genetic resistance to viruses (especially PPV) in *Prunus* spp. (rewied by Khalid et al., 2017). A popular case of virus resistance in *Prunus* spp., based on RNAi mechanism, is the C5 or Honeysweet transgenic plum clone resistant to PPV (Scorza et al., 2013). PPV-resistant Honeysweet plum was achieved using hypocotyl slices as starting material, which were transformed with the coat protein gene of PPV. After years of evaluations, it was demonstrated that the high resistance to PPV of the transgenic clone C5 exhibits the typical features of PTGS mechanism (Scorza et al., 2001). In particular, 24-nt long siRNAs were found out in the resistance clone when infected by PPV, which were considered responsible for the Honeysweet PPV resistance. The same approach could be attempted with other close-related species, such as peach, with the purpose of inducing PPV resistance.

In order to induce PTGS against PPV in *Prunus* spp., including peach varieties and rootstocks, it is essential to set reliable *in vitro* protocols for regeneration and genetic transformation. Unfortunately, *Prunus persica* L. is universally known as one of the most recalcitrant species in terms of genome modification and the production of transformed plants (Prieto, 2011). In general, transformation efficiency (number of transgenic shoots recovered in relation to explants initially transformed) depends on: (i) starting plant tissue; (ii) regeneration medium components/conditions; (iii) methods of transformation; (iv) selection method used to recover putatively transformed shoots (Srinivasan et al., 2004). *Agrobacterium*-mediated transformation is one of the main methods used to insert desired traits into dicotyledonous plant cells (Gelvin, 2000). When the intrinsic gene delivery ability of *A. tumefaciens* is exploited, various aspects need to be also evaluated, such as: (i) bacterial strain; (ii) antibiotics chosen to contain *A. tumefaciens* overgrowth after infection; (iii) reagents such as phenolics compounds and ethylene inhibitors commonly used to improve the bacterium gene transfer efficiency to the host cells (Opadobe, 2006; Petri et al., 2005).

In chapter 4, an *in vitro* regeneration protocol via organogenesis using leaf explants as starting material was optimized for the peach hybrid rootstock Hansen 536, to enable the insertion of gene constructs expressing traits of interest in this genotype, and in particular of a hairpin construct designed to silence PPV virus via PTGS. This construct was designed and generated starting from another hairpin construct against PPV, which was successfully validated by Pandolfini et al., (2003) in *N. benthamiana* model plant. Thus, the present study aimed to evaluate Hansen 536 transformation efficiency using leaf explants as starting explants for *Agrobacterium*-mediated genetic transformation trials; eGFP and/or kanamicyn selection systems were used in identifying putatively transformed events. Although the production of peach transformed plants through *A. tumefaciens* has been arduous to date, this transformation strategy

was used as first choice in this study. Later, a new strain of *A. rhizogenes* was also used as gene delivery system, considering that this *Agrobacterium* species has already been used successfully by our group in a preliminary study, which led to the production of Hansen 536 transgenic roots with high efficiency. Furthermore, the impact of 10 µM STS in the regeneration/selection medium was evaluated, considering that it was capable to significantly enhance regeneration from non infected leaf explants of Hansen 536 as it has been widely shown in chapter 4.

2. Materials and methods

2.1. Plant material

Apical expanding leaves from 3-week old elongated shoot cultures of the peach rootstock Hansen 536 (*P. persica x P. amygdalus*) were used as the source of explants. *In vitro* elongated shoots and leaf explants were obtained and prepared, respectively according to the protocols described in chapter 4. Briefly, the ready for-use starting explant for this study were young leaves along with their petioles, that were gently wounded on their abaxial surface (leaving the mid vein intact).

2.2. Agrobacterium species, gene constructs and leaves transformation conditions

The *A. tumefaciens* disarmed strain EHA105 and the *A. rhizogenes* disarmed strain 18r12v (Collier et al., 2016, 2018), both harbouring an hairpin gene construct designed to silence PPV virus, were used for transformation experiments. The *A. tumefaciens* disarmed strain EHA105 harboured the same gene construct already used in chapter 3. Briefly, the pK7WG2 binary vector (Karimi et al., 2002) expresses the PPV polyprotein hairpin and its constitutive promoter 35S of CaMV, and the *eGFP* plus the *nptII* genetic cassettes (the whole hairpin construct is referred to as *ihp35S-PPV194::eGFP* in this study). The same hairpin sequence was cloned in the pbin19 binary vector (Bevan, 1984), which confers resistance to kanamycin through NosPromoter-*nptII*-NosTerminator cassette. The gene construct (*ihp35s-PPV194::nptII*), kindly prepared by Dr. Molesini (University of Verona), was inserted in the *A. rhizogenes* disarmed strain 18r12v by electroporation (Sambrook, 1989). The *A. rhizogenes* disarmed strain 18r12v was kindly provided by Prof. Thilmony (U.S. Department of Agriculture).

The engineered *A. tumefaciens* strain EHA105 was grown and prepared for explant infection as described in chapter 3. Briefly, single colonies were inoculated in liquid YEB medium (5 g L^{-1} of yeast extract, 1 g L^{-1} of peptone, 5 g L^{-1} sucrose, 490 mg L^{-1} $MgSO_4$, pH 7.2) containing 152 µM spectynomicin and 61 µM rifampicin, and incubated overnight at 28°C with constant agitation (175 rpm) until reaching an $O.D._{600}$ of 1-1.2. The bacterial culture was pelleted (2500 x g for 15 min at 25°C) and re-suspended in MS basal salt and vitamins liquid medium including 20 g L^{-1} sucrose, 100 µM acetosyringone, pH 5.2. Then, this infection solution was shaken (175 rpm) at 24°C for 2 h before use. The explants were then dipped in the infection solution for 15 min, subsequently dried on sterile filter paper, and finally placed on co-culture medium (MS basal salt and vitamins, 30 g L^{-1} sucrose, 100 µM acetosyringone and 7 g L^{-1} plant agar, pH 5.2) for 48 h at 24 ± 1 °C in dark conditions. Preparation of the liquid culture of the engineered *A. rhizogenes* strain 18r12v and its preparation for explant infection was the same as for *A. tumefaciens* culture, except for the liquid medium for bacterial growth, corresponding to LB medium (5 g L^{-1} of yeast extract, 10 g L^{-1} of tryptone, 1 g L^{-1} glucose, 1 g L^{-1} NaCl, pH 7.2) supplemented with 152 µM spectynomicin and 105 µM kanamycin.

2.3. Kanamycin sensitivity tests on leaf explants and regenerating shoots of Hansen 536

Before starting with the transformation experiments, the toxicity threshold of kanamycin was established by culturing non-infected leaves of Hansen 536 in sterile plastic Petri dishes (9 cm x 1.5 cm) containing the previously optimized WPM 11 regeneration medium supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM) and increasing concentrations of kanamycin (0, 5, 10, 21, 31, and 42 µM). Leaves culture conditions were the same carried out following the procedures reported in chapter 4. Briefly, wounded abaxial surfaces of leaf explants were placed in contact with WPM 11 consisting of WPM basal salts and vitamins, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000, pH 5.7 supplemented with 15.5 µM BAP. Leaves were kept in darkness at 24 ± 1°C for three weeks and then moved to fresh medium and exposed to light (16-h photoperiod at light intensity of 40 µmol/m^2/s) at 24 ± 1°C for two weeks. For each selection condition, a total of fifty explants were prepared (ten leaves per Petri dish) and three independent trials were performed. Data on the leaf regeneration frequency espressed as percentage of leaves regenerating at least one shoot per total leaves treated and the average number of regenerating shoots per leaf were recoreded after seven weeks from the beginning of the culture. The same kanamycin sensitivity test was also carried out using WPM 11 supplemented with 10 µM STS as basal regeneration medium (the STS stock solution was prepared as described in chapter 4).

Furthermore, the impact on the health and propagation efficiency of single Hansen 536 regenerated shoots of different kanamycin concentrations added to the proliferation medium was evaluated. Shoot proliferation was performed according to the protocol previously described. Briefly, shoots regenerated from wild-type leaf explants were excised and proliferated on WPM medium, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000, pH 5.7 supplemented with 6.6 µM BAP and 0.1 µM NAA, and different concentrations of kanamycin (0, 10, 21, 42, 83 and 125 µM) for a total of three subcultures (2-week intervals). Shoot cultures were placed in the growth chamber under the same conditions described above. For each selection condition, twelve regenerated shoots were used, and three independent trials were carried out. The percentage of healthy and actively proliferating shoots expressed as [(number of proliferating shoots/total shoots treated) x 100] was acquired after six weeks from the beginning of elongation phase. In all the experiments described above filter-sterilized STS and/or kanamycin were added to the media after autoclaving and cooling down to 50°C.

2.4. Selection of GFP fluorescing calli and putative transgenic shoots regenerated on selection medium with or without STS after A. tumefaciens- or A. rhizogenes-mediated transformation

A total of 200 leaf explants were infected using *A. tumefaciens* strain EHA105 harboring the *ihp35S-PPV194::eGFP* gene construct. After the co-culture with *A. tumefaciens*, one half of leaf explants was placed in sterile plastic Petri dishes (ten leaves per dish) containing WPM 11 selection medium supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM) to contain *Agrobacterium* overgrowth, and 5 µM kanamycin. The remaining half of the leaf explants was placed on the same WPM 11 selection medium containing also 10 µM STS (ten leaves per Petri dish). In parallel, *Agrobacterium* non-infected leaf explants were used for regeneration frequency comparative purposes. A total of 200 non-infected leaf explants (used as regeneration positive controls) were split in two groups (100 leaves each) cultured on WPM 11 supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM), and with or without STS 10 µM. Another group of 200 non-inoculated leaf explants (used as regeneration negative controls) were split in two groups (100 leaves each) cultured on WPM 11 supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM) and 5 µM kanamycin, and with or without STS 10 µM.

Furthermore, a total of 200 leaf explants were infected using *A. rhizogenes* strain 18r12v harboring the *ihp35s-PPV194::nptII* gene construct. The whole experimental design (controls included) and the selection strategy applied were the same followed for the *A. tumefaciens* transformation experiments described above. All the leaf explants were kept in darkness at 24 ± 1°C for three weeks and then moved to fresh media containing 10 µM kanamycin (except for positive controls) and exposed to light (16-h photoperiod at light intensity of 40 µmol/m^2/s) at 24 ± 1°C for two weeks.

The *A. tumefaciens* infected explants were transferred on fresh media every two weeks, and the infected explants were observed under the stereomicroscope Leica MZ10F (Leica Mikrosystems GmbH, Wetzlar, Germany) to detect eGFP fluorescing calli and/or regenerating shoots (λ_{EX} = 480 nm and λ_{EM} = 510 nm). Images were acquired by the Leica DFC 450 C290 camera and processed through the Leica Application Suite V.4.5. (Leica Microsystems GmbH, Wetxlar, Germany). At each subculture, only explants showing eGFP fluorescence were transferred to fresh media.

All the transformation trials were repeated three times. Data on the leaf regeneration frequency expressed as percentage of leaves regenerating at least one shoot per total leaves treated and the average number of regenerating shoots per leaf were recorded for infected and non-infected explants after seven weeks from the end of co-culture period or beginning of the culture, respectively. Furthermore, only for *A. tumefaciens*-infected explants, the percentage of leaves showing eGFP fluorescing calli and/or shoots per total number of *Agrobacterium* infected leaf explants was annotated at 12 weeks post-infection.

2.5. Recovery of putative transgenic lines

Putative transgenic shoots regenerated from *A. rhizogenes* strain 18r12v-infected leaf explants were excised and cultured on WPM medium, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000, pH 5.7 supplemented with 6.6 µM BAP and 0.1 µM NAA for a total of three subcultures (2-week intervals) at 24 ± 1°C under a 16-h photoperiod at light intensity of 70 µmol/m^2/s. The above-mentioned elongation medium contained kanamycin at 10 and 21 µM for the first and second/third subculture, respectively. Single shoots (carefully labelled identifying the ones selected in presence of 10 µM STS) were then placed on kanamicyn-free rooting medium consisted of WPM basal salts and vitamins, 30 g L^{-1} sucrose and 5 g L^{-1} plant agar S1000, pH 5.7 supplemented with 0.5 µM BAP and 7.38 µM IBA for 20 days under the same conditions described above. *In vitro* rooted shoots were finally acclimatized in pots (7 x 7 cm) containing commercial peat and grown in the greenhouse.

2.6. PCR end-point of putatively transgenic lines

Genomic DNA was extracted from 100 mg of frozen young leaves from each putative transgenic and one wild-type lines (one plant for each line was randomly chosen) obtained from *A. rhizogenes* strain 18r12v-infected and wild-type leaves of Hansen 536, respectively using CTAB extraction method (Doyle, 1991). PCR analysis were performed to amplify 340 bp of constitutive promoter 35S coding sequence with the following primers: 35S-F, 5'-CTTCGTCAACATGGTGGAGCACGACA-3' and 35S-R, 5'-TGGGATATCACATCAATCCACTTG-3'. Furthermore, 456 bp of the *ihp-PPV194* gene sequence, which spans from the antisense arm of the hairpin sequence to the NOS terminator (NOS ter), were amplified with the following primers: hpPPV-F, 5'-TAGCTGTTGCACTCTCATATGTGTTT-3' and NOSTer-R, 5'-GGAAGGGACTGGCTGCTATTGGGCGAA-3'. The coding sequence of the translation elongation factor 2 (*Tef2* – 129 bp) was amplified from each sample and used as positive control (Tong et al., 2009). The PCR conditions were as follows: 95°C for 5 min; 40 cycles at 95°C for 1 min, 61.1°C for 1 min, and 72°C for 1 min; and 72°C for 5 min. The plasmid DNA of pbin19 ihp35s-PPV194::nptII was

used as positive control when both 35S and *ihp-PPV194* coding sequences were amplified. 10 µL of amplified fragments were loaded on agarose gel (1%, w/v) with SYBER Safe DNA Gel Stain (Invitrogen) and detected by UV after electrophoresis.

2.7. Statistical analysis

All the data shown here were transformed by the arcsine square root transformation, ARSIN (SQRT (X)), before analysis. The results acquired were analyzed by one-way ANOVA using Statistica 7 software (Statsoft Tulsa, CA, USA), and means were separated using the Newman–Keuls test ($p < 0.05$).

3. Results

3.1. Sensitivity to kanamycin for the optimization of the selection strategy

To define the concentration of kanamycin to apply for the selection of putative transformed events, non-infected leaf explants of Hansen 536 were placed on WPM 11 regeneration medium supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM) and increasing concentrations of kanamycin (0, 5, 10, 21, 31, and 42 µM). As expected, an increase in the concentration of the selective agent in the medium led to a progressive decline in the regeneration frequency as well as in the average number of regenerating shoots per leaf after seven weeks from the beginning of the culture (Figure 1 a, b). Furthermore, a gradual necrosis of the leaves and the regeneration of not healthy shoots were observed (Figure 1 c-h). In particular, the regeneration frequency as well as the average number of regenerating shoots per leaf significantly decreased more than two-fold (a mean of 16% ± 8.1 and 0.26 ± 0.09 shoots per explant, respectively) when kanamycin was used at a concentration of 5 µM compared to the control (a mean of 42% ± 7.3 and 0.66 ± 0.15 shoots per explant, respectively) (Figure 1 a, b). Moreover, shoot regeneration was almost completely arrested using concentrations of kanamycin equal or higher than 10 µM (Figure 1 a, b). In particular, a marked toxicity effect became evident after seven weeks of culture for the leaf explants cultured on kanamycin equal or higher than 21 µM (Figure 1 f-h). No significant differences in terms of kanamycin sensitivity have been observed when leaves were cultured on the same WPM 11 regeneration medium containing 10 µM STS (data not shown). Thus, a selection strategy characterized by an increasing concentration of kanamycin (from 5 up to 10 µM) was chosen as the proper selective strategy to be used in all transformation experiments, because it should be able to limit the regeneration of untransformed events, but at the same time should allow the survival, proliferation and eventually regeneration of putative transformed events.

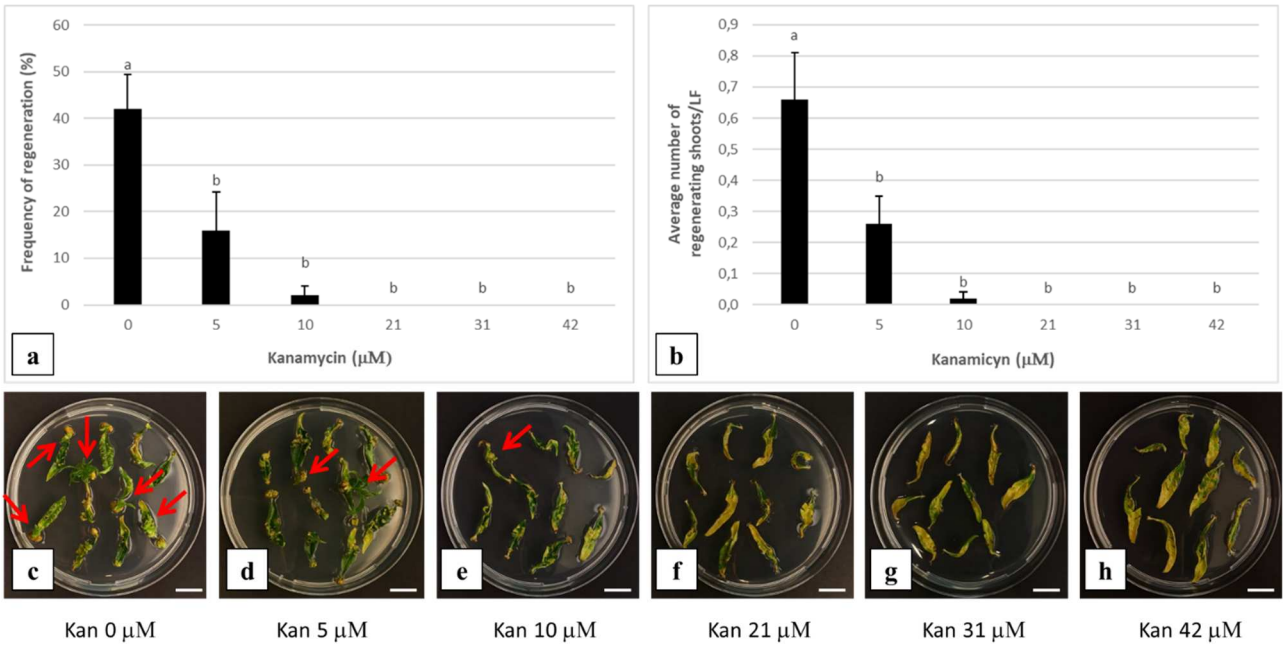


Figure 1. Kanamycin sensitivity test on non-infected leaf explants of Hansen 536: **(a)** Leaf regeneration frequency expressed as (number of leaf explants regenerating at least one shoot/total explants treated) x 100 and **(b)** the average number of regenerating shoots per leaf (LF) were recoreded after seven weeks from the beginning of the culture. The results were analysed by one-way ANOVA, and Newman-Keuls test ($p< 0.05$) ± SE ($n = 150$) was used to identify significant differences. Data reported in the pictures represent the mean ± SE of three independent experiments. **(c-h)** Non-infected leaf explants of Hansen 536 cultured on WPM 11 regeneration medium containing increasing concentrations of kanamycin (0, 5, 10, 21, 31, and 42 µM) after seven weeks from the beginning of the culture (bars = 1 cm). Red arrows indicate where adventitious shoot regeneration occurs.

Moreover, to establish the concentration of kanamycin for the selection of putative transformed events, shoots regenerated from control leaves of the above-mentioned experiments were cultured on propagation medium supplemented with increasing concentrations of kanamycin (0, 10, 21, 42, 83, and 125 µM). As expected, an increase in the concentration of kanamycin in the medium led to a progressive decline in the health of the shoots after six weeks from the beginning of the elongation phase (Figure 2 a, b). When kanamicyn was used at 10 µM, the percentage of healthy shoots decreased drastically to 41.7% (Figure 2 a). Moreover, shoots were almost completely died using concentrations of kanamycin equal or higher than 21 µM (Figure 2 a, b). Thus, to maintain a strict selective regime and to avoid the proliferation of "escapes", the use of 10 µM kanamycin for the first subculture, which increased up to 21 µM for the second and third subcultures, was established as the most suitable selective approach for the proliferation of all the putative transformed shoots.

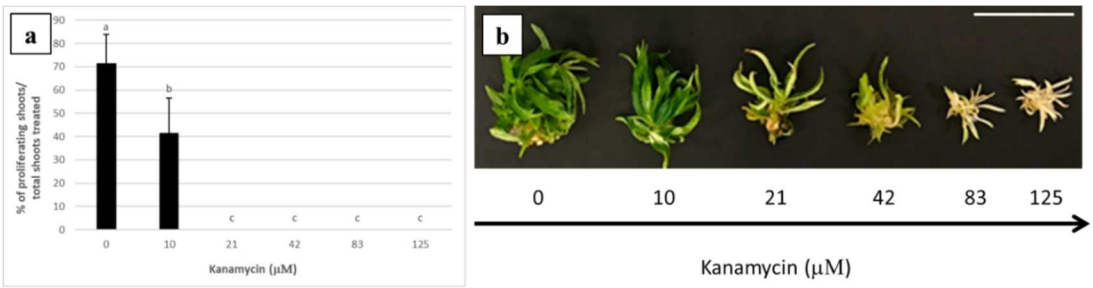


Figure 2. Kanamycin sensitivity test on shoots regenerated from leaves of Hansen 536: **(a)** The percentage of healty shoots expressed as (number of proliferating shoots/total shoots treated) x 100 was acquired after six weeks from the beginning of elongation phase. The results were analysed by one-way ANOVA, and Newman-Keuls test ($p< 0.05$) ± SE ($n = 36$) was used to identify significant differences. Data reported in the pictures represent the mean ± SE of three independent experiments. **(b)** Shoots regenerated from leaves of Hansen 536 cultured on proliferation medium containing increasing concentrations of kanamycin (0, 10, 21, 42, 83, and 125 µM) after six weeks from the beginning of the culture (bars = 1.5 cm).

3.2. Leaf transformation using A. tumefaciens strain EHA105

After *A. tumefaciens* infection, half of the leaf explants was placed onto WPM 11 selection medium supplemented with carbenicillin (238 µM) plus cefotaxime (210 µM), and 5 µM kanamycin, while the remaining half was placed onto the same selection medium supplemented with 10 µM STS. The infected-leaves were kept in darkness at 24 ± 1°C for three weeks and then moved to fresh media containing 10 µM kanamycin and exposed to light (16-h photoperiod at light intensity of 40 µmol/m^2/s) at 24 ± 1°C for two weeks. All the explants were then transferred on fresh media every two weeks and visual screening of fluorescence cells using eGFP as visual marker was performed at each subculture to identify the putative transformed events. Although the percentage of leaf explants showing eGFP fluorescence did not show significant differences when explants were cultured in the presence or absence of 10 µM STS, explants on both culture conditions continued to stably express eGFP after twelve weeks post infection (Figure 3 a). Parallely, positive controls (i.e non-infected leaves cultured on kanamycin free regeneration medium) presented a good regeneration potential (with data close to 50% in terms of regeneration frequency) (data not shown); despite this, with the applied protocol no transformed shoots have regenerated yet, but only eGFP fluorescing calli were observed (Figure 3 b, c).

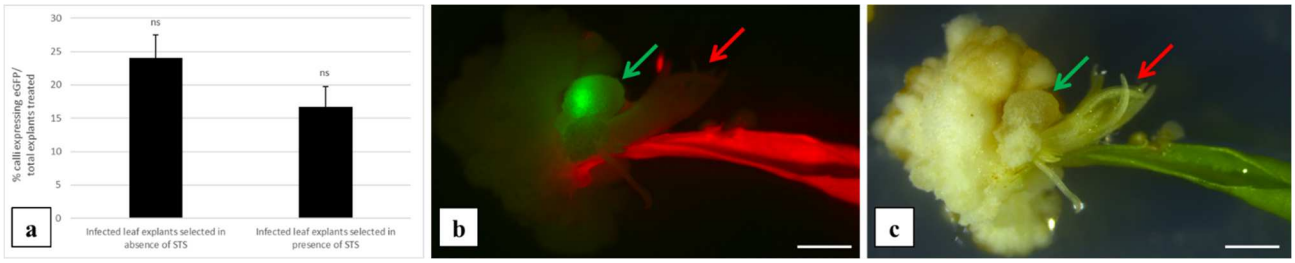


Figure 3. Transformation of Hansen 536 leaf explants using *A. tumefaciens* strain EHA105: **(a)** Percentage of leaf explants showing GFP fluorescing calli per total number of *Agrobacterium* infected leaf explants cultured for twelve weeks on selection media supplemented with or without 10 µM STS. The results were analysed by one-way ANOVA, and Newman-Keuls test ($p < 0.05$) ± SE (n = 300) was used to identify significant differences. Data reported in the picture represent the mean ± SE of three independent experiments. NS, not significant. **(b, c)** Hansen 536 stably transformed callus expressing eGFP (green arrow) and escape shoot (red arrow) observed under UV light **(b)** or under white light **(c)**. Photographs taken at five weeks post-infection (bar = 1 mm).

3.3. Leaf transformation using A. rhizogenes 18r12v

Similarly, leaf explants of Hansen 536 were infected using *A. rhizogenes* strain 18r12v, and their transformation competence was screened using only kanamycin selection. Three weeks after incubation in darkness, putative transformed shoots started to appear, mainly from the petiole surface (Figure 4 c). The regeneration frequency of infected leaf explants increased significantly compared to negative controls after seven weeks from the *Agrobacterium* infection, and no significant differences were observed between infected leaves cultured on WPM 11 selection medium compared to those cultured on the same medium containing 10 µM STS (Figure 4 a). As already shown in chapter 4, the average number of regenerating shoots per non-infected explant was higher (a mean of 1.17 ± 0.06 wild-type shoots per explant) when 10 µM STS was used in the regeneration medium compared to the other positive control (a mean of 0.61 ± 0.06 wild-type shoot per explant cultured in the absence of STS) after seven weeks from the beginning of the trial (Figure 4 b). Despite this, no significant differences in terms of the average number of regenerating shoots per explant were observed between infected explants cultured on WPM 11 selection medium compared to those placed on the same medium supplemented with 10 µM STS after seven weeks post-infection (Figure 4 b). Furthermore, the average number of regenerating shoots per infected leaf increased significantly (a mean of 0.18 ±

0.03 putative transformed shoots per explant) when STS was not used in the selection medium compared to its respective negative control (a mean of 0.02 ± 0.01 wild type shoots per explant) after seven weeks post-infection (Figure 4 b). Differently, the same value (a mean of 0.14 ± 0.03 putative transformed shoots per explant) obtained by selecting infected explants in presence of STS, was not significantly different to the average number of regenerating shoots per leaf obtained from its respective negative control (a mean of 0.04 ± 0.01 wild type shoots per explant) after seven weeks post-infection (Figure 4 b). Furthermore, leaf explants of Hansen 536 previously infected using *A. rhizogenes* strain 18r12v and cultured on the selection medium with or without 10 µM STS were capable to regenerate new adventitious shoots until 12 weeks post-infection (data not shown). Putative transformed shoots of approximately 1 cm in length (including few wild type shoots regenerated from positive controls) were transferred to elongation medium supplemented with kanamycin. A total of eleven putative transformed lines (six of them derived from selection in the presence of STS) proliferated and survived to the entire selection and proliferation procedure (Figure 4 d). Then they were successfully rooted and acclimatized to the greenhouse following our standard procedure described in the chapter five (Figure 4 e).

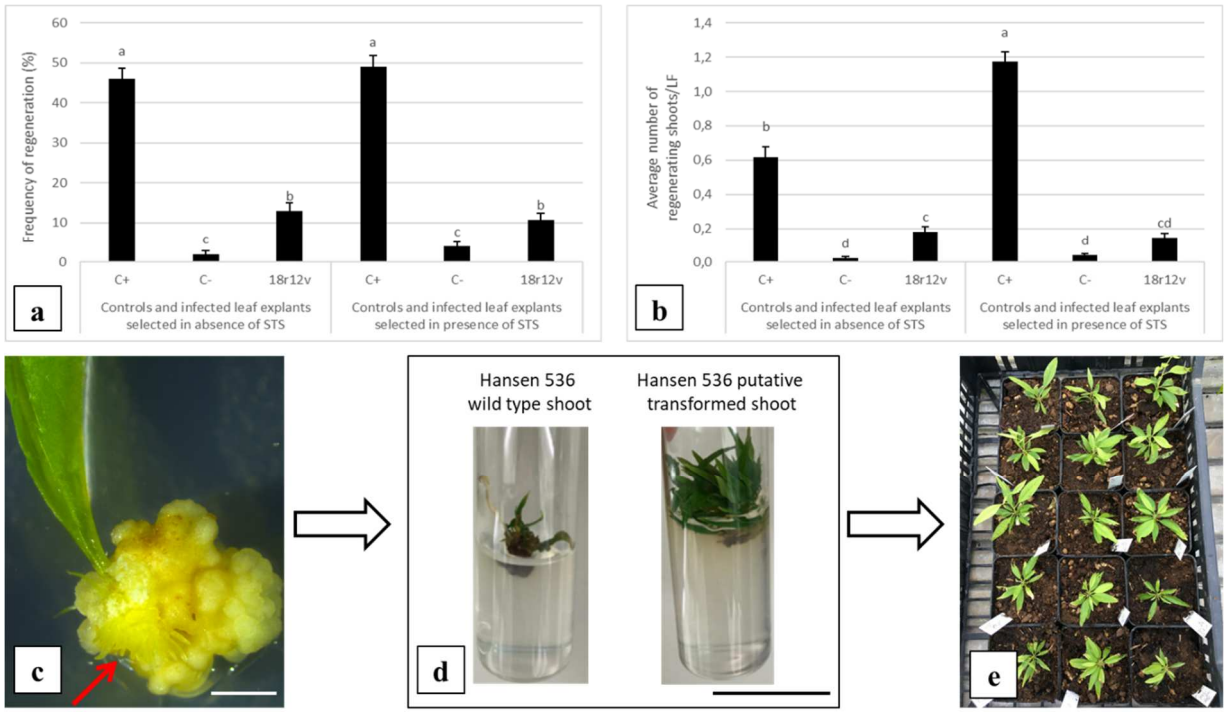


Figure 4. Transformation of Hansen 536 leaf explants using *A. rhizogenes* strain 18r12v: **(a)** Leaf regeneration frequency expressed as (number of leaf explants regenerating at least one shoot/total explants treated) x 100 and **(b)** the average number of regenerating shoots per leaf (LF) were recoreded after seven weeks post-infection. The results were analysed by one-way ANOVA, and Newman-Keuls test ($p < 0.05$) ± SE (n = 300) was used to identify significant differences. Data reported in the pictures represent the mean ± SE of three independent experiments. **(c)** Putative transformed shoot (arrow) regenerated from a leaf cultured onto selection medium in absence of STS after three weeks post-infection (bar = 1 mm). **(d)** Comparison between a wild-type shoot and a putative transformed shoot cultured on the same medium containing kanamycin after two weeks from the beginning of the proliferation phase (bar = 1.5 cm). **(e)** Acclimatized putative transgenic lines of Hansen 536 in 7 x 7 cm pots in greenhouse conditions.

3.4. PCR end-point of putatively transgenic lines obtained from A. rhizogenes strain 18r12v-infected leaves

Almost one year after the beginning of transformation trials, there were no transformed shoots regenerated from *A. tumefaciens* strain EHA105-infected leaves of Hansen 536, but eleven putatively transgenic lines (regenerated from *A. rhizogenes* strain 18r12v-infected leaf explants) were successfully proliferated and acclimatized to the

greenhouse. 100 mg of young leaves were collected from each putatively transformed and one wild-type lines of Hansen 536 (one plant for each line was randomly chosen) (Figure 5 a, b), which provided enough material for PCR analyses. After genomic DNA extraction, the insertion of ihp35s-PPV194::nptII sequence into the putatively transformed plants were checked by PCR end-point analysis. 340 bp of the constitutive promoter 35S coding sequence and 456 bp of the *ihp-PPV194* gene sequence were not successfully amplified from any of the putatively transgenic lines of Hansen 536. To date, PCR end-point using different pairs of primers and RT-PCR analysis to evaluate the insertion and the transcription of transgenes, respectively are still going.

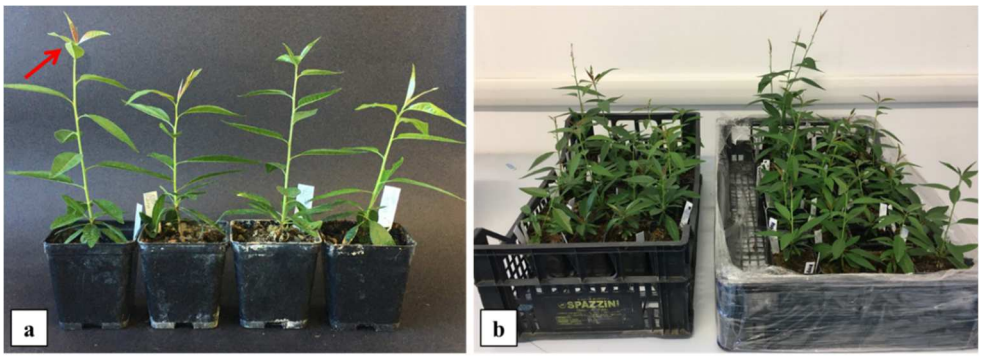


Figure 5. Putatively transgenic lines obtained from *A. rhizogenes* strain 18r12v-infected leaves: **(a, b)** Acclimatized putative transgenic lines of Hansen 536 in 7 x 7 cm pots used to perform PCR end-point analysis. The arrow indicates the pool of young leaves collected and used as source of genomic DNA.

4. Discussion

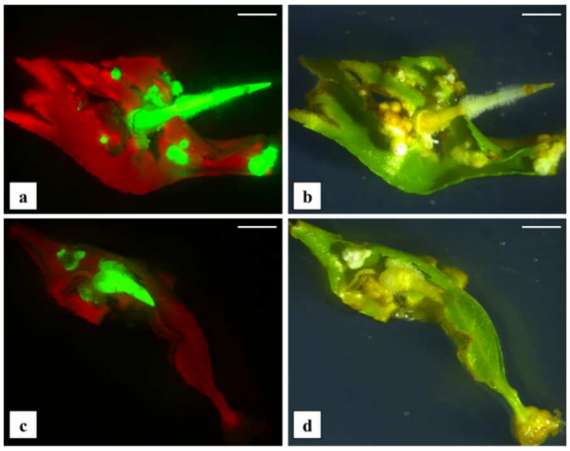
The regeneration protocol via organogenesis from leaf explants established as previously described was used for genetic transformation experiments with two different species of *Agrobacterium*, which contained the same hairpin gene constructs designed to silence PPV virus via PTGS, one expressing eGFP as reporter gene, and both expressing *nptII* gene as selectable marker. *ihp35S-PPV194::eGFP* gene construct has been already used in our previous study carried out on the peach hybrid rootstock Hansen 536, showing stable expression only in transgenic callus lines obtained from meristematic tissues (see chapter 3). With the purpose of obtaining transgenic shoots expressing this construct, a different starting explant and regeneration protocol were exploited for Hansen 536, and eGFP expression combined with kanamycin selection, as means of selection of putative transformed events after *A. tumefaciens* infection. However, this different regeneration system only led to the production of peach fluorescent proliferating calli and the regeneration of non-transformed shoots (escapes), applying a gradual selection strategy with kanamycin. In the current study, we obtained transgenic peach calli applying an "iterative" selection strategy, by using increasing kanamycin concentrations in the medium. A kanamycin concentration of 5 µM was used after the co-culture period, which is lower than the amount of kanamycin (21 µM) that completely inhibits the regeneration from Hansen 536 leaf explants (Figure 1 a). This choice was due to the fact that using high concentrations of antibiotic-selective agents at the beginning of the regeneration process may lead to a high rate of explant necrosis, which can affect also the survival of the few transformed cells (Petri et al., 2008). Three weeks after incubation in dark condition, when putative transformed shoots are supposed to appear, we increased the kanamycin concentration up to 10 µM. Furthermore, to avoid the regeneration of escape shoots, we frequently subcultured the explants (2-weeks interval), so to maintain them in contact with fresh medium and not-degraded selective agent (Rosellini et al., 2007). Sensitivity to the presence of the selective agent in the substrate has a huge impact on the adventitious regeneration frequency and therefore on the production of transgenic plants. Although a meticulous study has been carried out to optimize the suitable selection strategy using kanamycin, successful regeneration of transformed shoots has not been achieved yet starting from leaf explants of Hansen 536

inoculated with *A. tumefaciens* EHA105. In particular, only eGFP fluorescing calli and escape shoots were obtained, which were able to regenerate probably protected by callus cells resistant to kanamycin (Figure 3 b, c). Since the mid '70s, *A. tumefaciens* has been widely studied and exploited as a gene delivery system in plants; especially, *A. tumefaciens* strain EHA105 is one of the most used bacterium strains, considering its huge virulence and broad host-range (Chetty et al., 2013). Despite this, the current study confirmed the extremely low efficiency of *A. tumefaciens*-mediated transformation and the low level of correspondence between cells competent for transformation and those that have regeneration competence as the main bottlenecks to success in peach genetic transformation. As we have widely discussed in chapter 2, *A. rhizogenes*-mediated adventitious hairy root disease in dicotyledonous plants has been deeply studied and exploited as transgenic tissue generation system in plant, including peach (Hwang et al., 2017; Xu et al., 2020). During a preliminary experiment, an *A. rhizogenes* wild strain (not disarmed), kindly provided by Prof. Baraldi (University of Bologna) and harboring the pK7WG2 binary vector containing the reporter gene *eGFP,* was tested by us to infect Hansen 536 leaf explants, which led to the regeneration of fluorescent roots with high efficiency after three weeks post-infection (Supplementary Figure 1, and data not shown). The optimization of an adventitious shoot regeneration protocol using root as starting explant is very difficult, but the great potential of this species as gene transfer method has been immediately recognized. Thus, the use of *A. rhizogenes* disarmed strain as gene delivery system in peach avoiding root regeneration from starting explant seemed to be a more feasible approach. With the aim to obtain transgenic peach lines expressing a hairpin construct (*ihp35s-PPV194::nptII*) potentially capable of inducing PPV resistance via PTGS, kanamycin selection on regenerating shoots from leaf explants previously infected with *A. rhizogenes* strain 18r12v was applied, and eleven putative transgenic lines of the peach rootstock Hansen 536 were isolated and analyzed.

In order to investigate also the influence of STS on the regeneration of Hansen 536 putative transformed lines, half of the infected leaf explants were placed on selection medium containing 10 μM STS, the optimal concentration which significantly improved shoot regeneration by increasing two-fold the average number of shoots per leaf explant in our previous regeneration study reported in chapter 4. Although the average number of regenerating shoots per non-infected explant was higher when 10 μM STS was used in the regeneration medium compared to the other positive control cultured in the absence of STS after seven weeks from the beginning of the trial (Figure 4 b), data recorded from infected explants using *A. rhizogenes* strain 18r12v showed that STS as ethylene inhibitor added to selection medium did not improve regeneration rates compared to the same explants selected in a medium without STS after seven weeks post-infection (Figure 4 b).

In conclusion, even if molecular analysis needs to be completed to confirm the presence or the absence of transgenes in these eleven putative transformed lines of Hansen 536, to the best of our knowledge this represents the first attempt of producing transformed peaches using *A. rhizogenes* strain 18r12v, and a promising strategy to genetically transform other close-related species, such as apricot, Japanese plum or cherry, which are all recalcitrant to *A. tumefaciens*.

5. Supplementary material



Supplementary Figure 1. Transformation of Hansen 536 leaf explants using *A. rhizogenes* strain wild: Hansen 536 transformed roots expressing eGFP under UV light **(a, c)** and under white light **(b, d)** after three weeks post-infection (bar = 1 mm).

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CHAPTER 7

RESEARCH SECTION

TOPICAL APPLICATION OF RNAi-INDUCING DOUBLE-STRANDED RNA MOLECULES FOR NON-TRANSGENIC RESISTANCE TO PPV VIRUS IN THE PEACH ROOTSTOCK GF305

1. Introduction

The previous chapters described the results of different strategies aimed at producing transgenic peaches. Specifically, we focused our attention on the use of RNAi-based gene constructs to introduce stable resistance in peach plants against PPV virus, by triggering PTGS. Recently, the topical application of RNAi-inducing double stranded RNA (dsRNA) to plants, has been considered as an alternative option to the stable production of dsRNA in crops (HIGS strategy) for virus resistance (Dalakouras et al., 2020). This method also represents a potential tool for pathogen and pest defence for those species with high recalcitrance to genetic transformation systems, as peach.

Concerning the exogenous application of dsRNA (SIGS strategy) used to induce the resistance in plant against target viruses, various aspects have to be taken into consideration. For large-scale application of dsRNA molecules, such as for greenhouse and/or open field environments, efficient and low-cost production systems for their large-scale production are required. *In vitro* enzymatic synthesis and *in vivo* production strategies by using bacteria are the two main options to produce dsRNA. The *in vitro* synthesis of dsRNA consists in the enzymatic transcription of two complementary ssRNA strands (followed by physical annealing) using the DNA-dependent RNA polymerases (DdRP) of the bacteriophage T7 (Carbonell et al., 2008; Tenllado et al., 2001). For plant virus control, target sequences are transcribed by DdRPs from cDNAs template extracted from plants infected by the target virus, using primers that carry the T7 promoter at their 5'-end; alternatively the *in vitro* transcription by DdRP can occur starting from plasmids carrying the target viral sequences cloned between two T7 promoters (Konakalla et al., 2016). As widely reviewed in the first chapter, different kits are commercially available with this aim. Moreover, *in vivo* methods using bacteria have been optimized using *E. coli* (Tenllado et al., 2003; Yin et al., 2009) or *P. syringae* (Aalto et al., 2007; Niehl et al., 2018). For example, in the *E. coli* system, a stable replicating plasmid carrying the target viral sequence cloned within two T7 promoters is introduced into bacterial cells. Chemical induction of the T7 DdRP gene, which is expressed by a gene cloned in an additional plasmid, follows and the target sequences are transcribed in both directions. Then, the newly generated ssRNA molecules anneal, producing the desired dsRNAs. Their degradation is inhibited using *E. coli* RNase-III deficient strains. However, most studies reporting the topical application of dsRNAs produced *in vitro* or *in vivo* showed the low stability of these molecules since their antiviral effects lasts for a few days. As extensively reviewed in the first chapter, dsRNA formulations based on biocompatible materials are being investigated; these include delivery of dsRNA into virus or virion-like particles (Dalakouras et al., 2020; Zotti et al., 2018) and/or layered double hydroxide (LDH) clay nanosheets (BioClay) (Mitter et al., 2017). Another delivery method, which seems to be suitable to trigger virus resistance in plants, is the high-pressure spraying system able to induce a symplastic transport when small RNAs (22-nt or 24-nt asymmetric) are exogenouly applied on the plant tissues (Dalakouras et al., 2020).

In this chapter, we present other procedures undertaken at our facilities aimed at obtaining non-transgenic resistance to PPV in the peach rootstock GF305. In particular, we describe easy systems for the production and topical application of dsRNA molecules against PPV in peach.

2. Materials and methods

2.1. Plants

Wild-type *Nicotiana benthamiana* plants were grown in commercial peat in 14 cm wide pots under greenhouse conditions at a mean temperature of 24°C under natural light. Plants were grown until they had approximately ten leaves and then used for agroinfiltration procedure. Peach rootstock GF305 plants (provided by Centro Attività Vivaistiche, Faenza, Italy) were cultivated in 21 cm wide pots in an insect-proof greenhouse (provided by Centro Ricerche Produzioni Vegetali, Cesena, Italy) at a mean temperature of 24°C under natural light. One-year-old GF305 plants were used for inoculation with PPV virus and dsRNAs.

2.2. Agrobacterium strains and gene constructs

A. tumefaciens disarmed strains, harbouring gene constructs designed to silence different plant viruses (such as PPV virus, GFLV and GLRaV viruses), were used for agroinfiltration procedure. The *A. tumefaciens* disarmed strain GV2260 harboured a derivative of pBin19 binary vector (Bevan, 1984) expressing the hairpin gene construct *hp-GFLV-GLRaV* and was used as control in this study. The *A. tumefaciens* disarmed strain EHA105 harboured the same gene construct already described and used in chapter three. Briefly, the pK7WG2 binary vector (Karimi et al., 2002) expresses the PPV polyprotein hairpin and its constitutive promoter 35S of CaMV, and the *eGFP* plus the *nptII* genetic cassettes (the whole hairpin construct is referred to as *ihp35S-PPV194::eGFP* in this study). Lastly, the *A. tumefaciens* disarmed strain GV2260 harboured a derivative of pBin19 binary vector (Bevan, 1984) expressing the above-mentioned PPV polyprotein hairpin and its constitutive promoter 35S of CaMV, and the *nptII* genetic cassette (the whole hairpin construct is referred to as *ihp35s-PPV194::nptII* in this study). Prof. Pandolfini and Dr. Molesini (University of Verona), kindly provided *A. tumefaciens* disarmed strains GV2260, harbouring gene constructs described above.

2.3. Agroinfiltration procedure

The agroinfiltration protocol reported by Voinnet et al. (2003) was used including slight modifications. The engineered *A. tumefaciens* strain EHA105 was grown as described in chapter three. Briefly, single colonies were inoculated in liquid YEB medium (5 g L^{-1} of yeast extract, 1 g L^{-1} of peptone, 5 g L^{-1} sucrose, 490 mg L^{-1} $MgSO_4$, pH 7.2) containing 152 µM spectynomicin and 61 µM rifampicin, and incubated overnight at 28°C with constant agitation (175 rpm) until reaching an OD_{600} of 1.5. The bacterial culture was pelleted (5000 x g for 15 min at 4°C) and re-suspended in the infiltration liquid medium ($MgCl_2$ 10 mM and acetosyringone 150 µM), to an OD_{600} of 0.3. Cells were left in this medium for 2 h at room temperature without shaking before infiltration. Growth of liquid cultures of the two-engineered *A. tumefaciens* strain GV2260, and their preparation for agroinfiltration procedure were the same as for *A. tumefaciens* strain EHA105, except for antibiotics used for bacterial growth, corresponding to 105 µM kanamycin, 122 µM rifampicin and 175 µM streptomycin. Approximately 400 µL of *Agrobacterium* mixture was gently infiltrated into the abaxial side of *N. benthamiana* young leaf using an insulin syringe (Figure 1 a). The mixture was gradually infiltrated, covering the whole surface of the leaf (Figure 1 b). A total of six plants per each *Agrobacterium* mixture were used and a total of three young leaves (carefully labelled to identify the ones treated) per each plant were infiltrated. Transient expression was assayed three and five days after infiltration. All the plants were maintained in the greenhouse for the duration of the experiment.

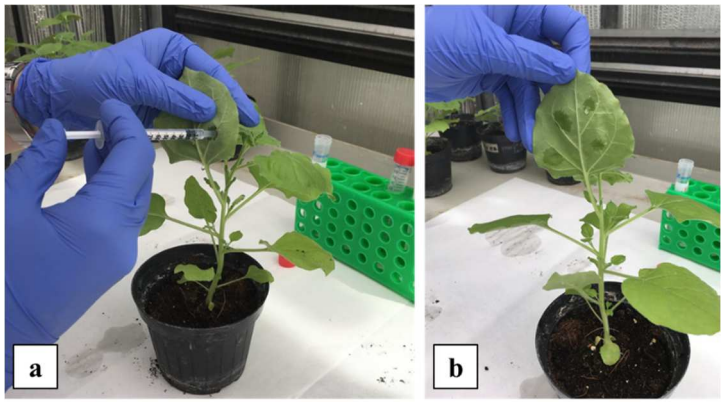

Figure 1. Agroinfiltration procedure in *N. benthamiana* plants: **(a)** Approximately 400 µL of *Agrobacterium* mixture was gently infiltrated into the abaxial side of *N. benthamiana* young leaf using an insulin syringe; **(b)** the mixture was gradually infiltrated, covering the whole surface of the leaf.

2.4. RT-PCR for dsRNA detection within the agroinfiltrated leaves

Total RNA was extracted from 100 mg of frozen leaves (pool) using the commercial kit Nucleospin RNA plant (Macherey-Nagel) following the manufacturer instructions. Samples were treated with DNase to clean them from any genomic DNA contamination, and 1 µg of total RNA was primed with oligo(dT)$_{15}$ and reverse transcribed using Goscript™ Reverse Transcription System (Promega) according to the manufacturer instructions. For the expression analysis of both gene constructs containing the PPV polyprotein hairpin, 250 bp of the *ihp35S-PPV194* gene sequence, which spans from the InLAX intron to the antisense arm of the hairpin sequence, was amplified with the following primers: InLAX-F, 5'-GGTACCGTACAAATTTCATTTTTTTTTCTTATAACA-3' and hpPPV-R 5'-GGATCCACTGCAAGTCAAGATGTCAACCATTGTATTTGGCTCATTC-3'. The PCR conditions were as follows: 95°C for 5 min; 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. cDNA from plants expressing the hairpin gene construct *hp-GFLV-GLRaV* was used as negative control. 5 µL of amplified fragments were loaded on agarose gel (2%, w/v) with SYBER® Safe DNA Gel Stain (Invitrogen) and detected by UV after electrophoresis.

2.5. Extraction and topical application of dsRNA

A total of 24 *N. benthamiana* plants were used in a second round of agroinfiltration following the protocol previously described in order to provide dsRNA for topical application (Figure 2). One half of tobacco plants was inoculated using *A. tumefaciens* GV2260 harbouring *hp-GFLV-GLRaV* gene construct. The remaining half of the plants was inoculated using *A. tumefaciens* EHA105 harbouring *ihp35S-PPV194::eGFP* gene construct. Five days after infiltration, dsRNA was extracted from 100 mg of frozen leaves (pool) using the commercial kit *mir*Vana™ miRNA Isolation (Invitrogen) following the manufacturer instructions. dsRNA samples were placed in dry ice just prior to use in the greenhouse. In the late spring of 2018, one-year-old plants of GF305 (a peach hybrid rootstock highly susceptible to PPV) were inoculated through the 'chip-budding' grafting method with a PPV infected bud of dormant shoots collected from peach plants showing strong PPV symptoms, available in the screen-house dedicated to the peach-PPV monitoring program at Centro Ricerche Produzioni Vegetali, Cesena, Italy. Different concentrations of naked dsRNA (Table 1) were dispensed in the infected buds with a pipette just prior grafting (Figure 3). Thus, treated plants were kept in an insect-proof greenhouse at a mean temperature of 24°C under natural light for four months. Later, plants were subjected to vernalization at 7°C for two months, and then moved back to the greenhouse for other four months at the same conditions previously described. In the late spring of 2019 (approximately eleven months after the beginning of

the experiment), plants were inspected for Sharka symptoms, and PPV was detected using DAS-ELISA. A total of five plants (positive controls included) per each dsRNA concentrations were used (Table 1). In addition, a total of five healthy GF305 plants were used as witness plants in this study.

Figure 2. Agroinfiltrated *N. benthamiana* plants used as source of dsRNA.

Table 1. Topical application of naked dsRNA on infected GF305 plants

Treatment	*Agrobacterium* strain infiltrated in *N. benthamiana*	Gene construct expressed in *N. benthamiana*	dsRNA used for topical application	dsRNA concentration dispensed in the infected bud
1	*A. tumefaciens* EHA105	*ihp35S-PPV194::eGFP*	PPV dsRNA	800 ng
2	*A. tumefaciens* EHA105	*ihp35S-PPV194::eGFP*	PPV dsRNA	1.6 µg
3 (positive control)	*A. tumefaciens* GV2260	*hp-GFLV-GLRaV*	GFLV-GLRaV dsRNA	800 ng
4 (positive control)	*A. tumefaciens* GV2260	*hp-GFLV-GLRaV*	GFLV-GLRaV dsRNA	1.6 µg

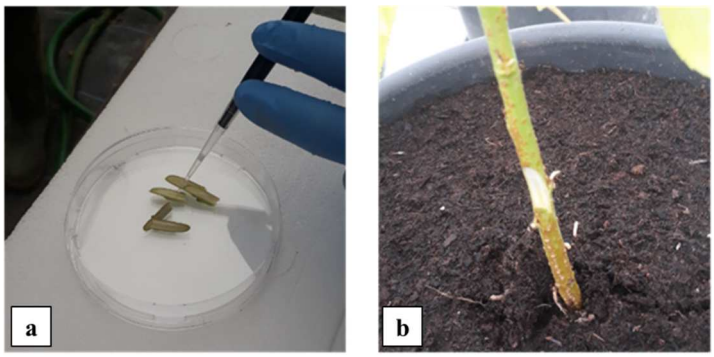

Figure 3. Topical application of dsRNA in GF305 plants: **(a)** Naked dsRNAs were dispensed in the infected bud with a pipette **(b)** just prior grafting.

2.6. DAS-ELISA for PPV detection within GF305 plants

To verify the presence or absence of PPV in the treated GF305 plants, a DAS (double-antibody sandwich)-ELISA (polyclonal antibody and alkaline phosphatase-conjugated polyclonal antibody) assay was carried out (Adams, 1978) using the reagents provided by LOEWE Biochemica GmbH (Germany). Leaves grown just above the rootstock-scion junction were collected from each plant eleven months after the PPV inoculation and dsRNA topical application. Leaf tissues were ground (1:5 w/v) in extraction buffer, and then the supernatant was used in the test with rabbit

polyclonal antibodies against the coat protein of PPV (Anti-Virus-IgG, Anti-Virus-IgG-AP-conjugate). Absorbance values at 405 nm were determined after 1-2 h using a Microplate Reader 550 (Biorad). According to Sutula (1986), samples with optical densities double that of the healthy controls were considered ELISA-positive.

3. Results

3.1. Production of RNAi-inducing dsRNA against PPV through agroinfiltration

Agroinfiltration procedure was used to produce RNAi-inducing dsRNA against PPV virus in this study. Three and five days after infiltration, total RNA was extracted from different pools of leaves collected from *N. benthamiana* plants, which were infiltrated with *A. tumefaciens* strains EHA105 and GV2260 harbouring different type of gene constructs. In particular, the transcription of PPV polyprotein hairpin sequence into agroinfiltrated *N. benthamiana* plants was checked by RT-PCR (Figure 1). 250 bp of the *ihp35S-PPV194* sequence was successfully amplified from *N. benthamiana* plants, which were infiltrated with *A. tumefaciens* strain EHA105, confirming the transient expression of the *ihp35S-PPV194::eGFP* gene construct three days after infiltration (Figure 1). Furthermore, the transcription of PPV polyprotein harpin sequence was observed also five days after infiltration of tobacco plants using both *A. tumefaciens* strains GV2260 and EHA105 (Figure 4). Generally, the results obtained showed a higher transient expression of the PPV polyprotein hairpin sequence five days after infiltration using *A. tumefaciens* strain EHA105 (Figure 1).

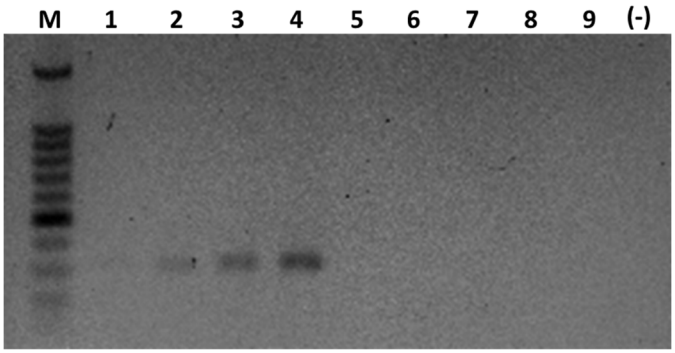

Figure 4. RT-PCR analysis of *ihp35S-PPV194* (250 bp) sequence of six different pools of leaves collected three and five days after infiltration of *N. benthamiana* plants using *A. tumefaciens* strains EHA105 and GV2260. M, DNA marker (100 bp DNA Ladder, Invitrogen, Carlsbad, CA, USA). Lanes 1 and 2 shows the PCR results using total RNA extracted from two different pools of leaves collected three days after infiltration of tobacco plants using *A. tumefaciens* strain GV2260 and EHA105, respectively. Lanes 3 and 4 shows the PCR results using total RNA extracted from two different pools of leaves collected five days after infiltration of tobacco plants using *A. tumefaciens* strain GV2260 and EHA105, respectively. Lanes 7 and 8 shows the PCR results using total RNA extracted from tobacco leaves expressing the hairpin gene construct *hp-GFLV-GLRaV* three- and five-days post-infiltration, respectively (negative controls). The lane labelled "(-)" shows the PCR result using water as negative control. Lanes 5, 6, and 9 were not used.

3.2. Topical application of RNAi-inducing dsRNA against PPV in the peach rootstock GF305

Five days after infiltration, dsRNA was extracted from 100 mg of leaves with a yield of approximately 40 ng/µL. Thus, one-year-old GF305 plants were inoculated with PPV and increasing concentrations of naked RNAi-inducing dsRNA against PPV. Results obtained from the greenhouse experiment showed that none of the concentrations tested was able to prevent PPV infection in the peach rootstock GF305. Eleven months after the beginning of the experiments, PPV symptoms were observed including vein chlorosis and some distortions on the leaves (Figure 5 a, b). As shown by ELISA, no obvious differences were observed among treatments (positive controls included). Generally, optical densities were approximately 0.065 and 3.085 in non-inoculated (healthy witness plants) and infected symptomatic GF305 plants, respectively (data not shown).

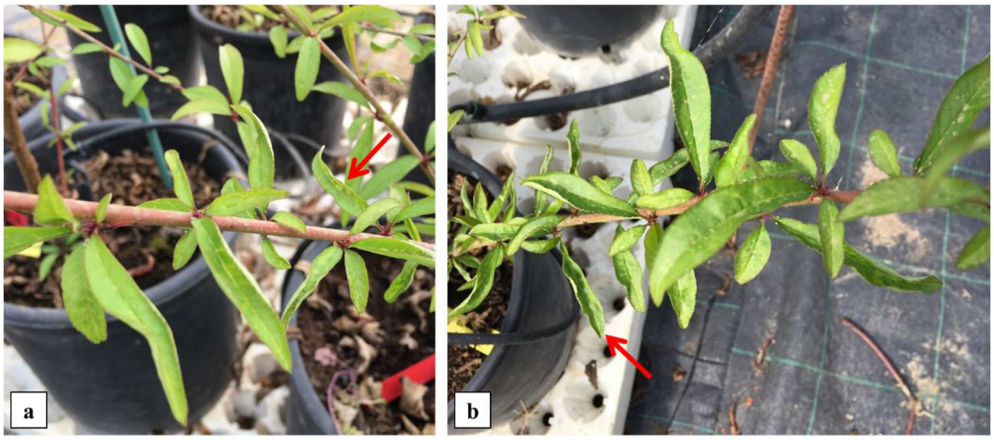

Figure 5. Inspection for Sharka symptoms on GF305 treated plants: **(a)** Vein chlorosis (arrow) and **(b)** distortion (arrow) on leaves of GF305 plants, which were subjected to treatment 1. Images were taken eleven months after the beginning of the experiment.

4. Discussion

A preliminary attempt to induce non-transgenic resistance to PPV virus in GF305 plants was carried out in this study. GF305 is characterized by its high susceptibility to PPV (Bernhard et al., 1969), and is used as rootstock in PPV resistance evaluation assays in *Prunus* spp. (Audergon et al., 1995; Gabova, 1994; García-Almodóvar et al., 2014; Martinez-Gomez and Dicenta, 2000; Rubio et al., 2009, 2013). The resistance evaluation process in controlled environment, usually carried out in insect-proof greenhouse, is a complicate procedure that requires the development of specific protocols, in controlled screen-houses, for testing the complex host-virus interaction and the plant response to the infection (Audergon et al., 1995; Martinez-Gomez and Dicenta, 2000; Moustafa et al., 2001). In particular, the evaluation procedure in controlled conditions includes various steps such as identification of the virus infection source, the inoculation of the plant followed by an artificial cold treatment to accelerate the infection process and then the evaluation of the susceptibility/resistance response by visually monitoring for PPV symptoms and detection strategies like ELISA (Rubio et al., 2009).

On the other side, for the exogenous application of dsRNA in greenhouse-controlled conditions, efficient and low-cost procedures for its large-scale production are essential. In the present study, an easy system to produce RNAi-inducing dsRNA against PPV has been developed through the transient expression of these dsRNA molecules induced by *A. tumefaciens* in *N. benthamiana* plants. Agroinfiltration, the direct injection of a specific agrobacterial solution to the young leaves of several plant species, has been described as an efficient system to analyze small RNA, protein localization and antibody production (Goodin et al., 2002; Rodríguez et al., 2005). The *Agrobacterium*-mediated transient expression has been reported in several plant species including *N. benthamiana* (Liu et al., 2010). There are several advantages in using tobacco plant for agroinfiltration such as its easy management in all plant research laboratories. Furthermore, the procedure is simple and fast; no expensive equipment is required, and the whole process needs no more than one week. Nevertheless, in view of the experience gained in this study, agroinfiltration does not appear as the best option for dsRNA production, considering that yields of these RNAi effective molecules are relatively low (40 ng dsRNA/µl) using the extraction kits commercially available. *In vitro* enzymatic synthesis using specific kits and *in vivo* approaches using bacterial cells are the two main alternatives of dsRNA production, since they are feasible in research laboratories and characterized by high performances in terms of yields. Moreover, different companies able to a low-cost mass production of dsRNA of interest are now available (RNAgri, agroRNA, GreenLight biosciences, nanoSUR) (Taning et al., 2020).

For the exogenous application of dsRNA molecules in plants, suitable methods for its delivery are required as well. In the current study, an easy method for the delivery of dsRNA has been attempted with poor results. Generally, an important aspect of dsRNA use for viral resistance in plants is to supply systemic protection rather than local protection; unfortunately topical application is limited to the adsorption of dsRNA into the treated cells, leaving all the others unprotect (Uslu and Wassenegger, 2020). However, the plant cell wall is a hard obstacle to pass through and only few authors declare that topical application of naked dsRNA induces RNAi in plants (Dubrovina et al., 2019; Mitter et al., 2017). Anyway, the lack of molecular fingerprints of RNAi such as the existence of small interfering RNA (siRNA) upon exogenous dsRNA application raises questions about the working mechanism of the exogenous dsRNA (Uslu and Wassenegger, 2020). It has been demonstrated that silencing molecules in grafting experiments are able to move along the phloem (Kehr and Kragler, 2018). Therefore, insertion of dsRNA into the vascular system is crucial for systemic delivery and thus, for setting up viral resistance. In order to transport naked dsRNA into *N. benthamiana* cells, Uslu et al. (2020) used the high-pressure spraying technique, already demonstrated to lead to local and systemic silencing in *N. benthamiana* when siRNAs are used (Dalakouras et al., 2016, 2018, 2020). In this study, the authors showed that high-pressure-sprayed naked dsRNAs were not processed into specific siRNAs by RNAi machinery and then they did not induce silencing suggesting that dsRNAs were insufficiently taken up by tobacco cells (Uslu et al., 2020). Despite being a promising strategy for plant protection against viruses, the working mechanism of the exogenous dsRNA remains controversial and mysterious to date.

In conclusion, as the exogenous application of dsRNA to stimulate the RNAi pathway against target viruses in plants is a promising option to protect plants from virus infections in the near future, the development of methods, which improve both production, and delivery of dsRNA has become a major challenge. This application could have a high impact in controlling the infection on orchards with susceptible cultivars as soon as the diseases start to spread.

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GENERAL CONCLUSIONS

Sharka disease represents a major threat to peach production worldwide. The use of conventional breeding techniques for obtaining PPV resistance in peach is mainly limited by its heterozygosity, long reproductive cycle, and multiple gene and/or recessive inheritance. To overcome these limitations, biotechnological strategies exploiting RNAi mechanism are considered promising tools to induce PPV resistance in peach and other *Prunus* species.

With the aim to stably express RNAi-inducing dsRNA against PPV in peach (HIGS strategy), this thesis was focused on developing of efficient and reproducible *in vitro* protocols for regeneration and genetic transformation suitable for this species. The results presented here confirm that adventitious shoot regeneration does not seem to be the main problem for this species. Even if with different regeneration rates, we showed adventitious shoot regeneration via organogenesis starting from different mature and juvenile tissues such as meristematic bulks, expanding leaves, hypocotyls and cotyledons of different peach genotypes including rootstocks and cultivars. Furthermore, even though somatic embryos have not been obtained by us using adult tissues, cream-colored calli formation in expanding leaves and anthers of different rootstocks and cultivars was observed and considered as promising starting point for further SE induction experiments. On the other side, the integration of the transgene/s into the plant genome and then the recovery of transformed plants is extremely difficult. To the best of our experience, there should be an overlap between peach tissue cells able to regenerate adventitious shoots and those amenable for transformation, in order to successfully produce transgenic plants. Although the first successful attempt of producing transformed peach calli has been reported, transformed plants starting from Hansen 536 meristematic bulks and expanding leaves have not been obtained yet by using *A. tumefaciens* disarmed strain EHA105. Even if molecular analysis needs to be completed to confirm the presence or the abscence of transgenes in the eleven putative transformed lines obtained from Hansen 536 expanding leaves, the use of *A. rhizogenes* disarmed strain 18r12v could be an appealing approach for peach genetic improvement, as the production of peach transgenic plants through *A. tumefaciens* has been arduous to date.

Recently, the exogenus application of RNAi-inducing dsRNA to plants, has been considered as an alternative option to the transgenic resistance in crops against viruses. With the aim to obtain non-transgenic resistance to PPV in the peach rootstock GF305 (SIGS strategy), this thesis was also focused on development of systems for the production and topical application of RNAi-inducing dsRNA against PPV in peach. Although the procedures used have been simple, fast, and cheap, the agroinfiltration and the molecules delivered system used did not seem to be the best choices due to the low yield in terms of product and very low chance to reach the phloem, respectively. Essentially, the SIGS approach shown here is at a very preliminary stage and alternative strategies in terms of both dsRNA production and delivery must be explored in the next future.

In conclusion, the overall research work has improved the knowledge on factors controlling peach regeneration also in post- *Agrobacterium* infection conditions, especially when both meristematic and leaf tissues were used as starting material. The development of an efficient peach transformation system is needed to use new biotechnological tools including RNAi, which may be also induced by the exogenous application of dsRNA, in order to obtain virus resistance in plant. The development of new dsRNA products can be a solution able to control PPV infection in stone fruits, but a specific research program has to be conceived in oder to identify the most appropriate formulates for increasing the efficiency of dsRNA targeting PPV genes and their delivering methods.

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